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Characterization of the Subunits of the McrBC Restriction System in Escherichia Coli K12.

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CHARACTERIZATION OF THE SUBUNITS OF THE MCRBC RESTRICTION SYSTEM IN *E. COLI* K12

A Dissertation

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DEDICATION

This dissertation is dedicated to my loving wife, Dian, and our soon to be born baby.

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ABSTRACT

The McrBC (Modified Cytosine Restriction) restriction system has the ability to restrict DNA containing 5-hydroxymethylcytosine, N^4-methylcytosine, and 5-methylcytosine at specific sequences. The *mcrB* gene produces two gene products. The complete *mcrB* open reading frame produces a 53-kDa protein ($McrB_L$) and a 35-kDa protein ($McrB_S$). The smaller McrB polypeptide is produced from an inframe, internal translational start in the *mcrB* gene. The *mcrC* gene produces a single 38-kDa protein.

Evidence was presented that $McrB_S$ regulates the activity of McrBC. When $McrB_S$ was overproduced in a $McrBC^+$ background, there was dramatic loss of restriction. Underproduction of this protein using antisense RNA caused variable restriction and triggered the SOS response indicating extensive DNA damage. Based on experimental results, $McrB_S$ was found to have distinct interactions with McrC and $McrB_L$. A unique assay, termed the restriction rescue assay, was used to examine $McrB_S$-McrC binding. Truncated versions of $McrB_S$ were used in the McrB* assay to provide evidence that $McrB_S$ and $McrB_L$ bind. In some cases, elevated levels of McrB* activity, hyper-restriction, observed in the presence of $McrB_S$ peptides was accompanied by induction of SOS, slow growth and cell death. Based on findings, we propose that the active restriction complex minimally consists of a $McrB_L$ homodimer. $McrB_S$ regulates restriction by binding $McrB_L$ to form an inactive $McrB_L$-$McrB_S$ heterodimer. McrC associates with the active $McrB_L$ homodimer to form the McrBC endonuclease and with $McrB_S$ containing inactive

complexes. Protein-DNA binding studies and *in vitro* cleavage studies allowed us to define specific binding site, cofactor, and subunit requirements for this restriction system. Results of these assays suggest that no subunit alone can bind specifically to DNA containing methylated *Pvu*II sites. Target DNA fragments containing methylated *Pvu*II sites were specifically bound when all three McrBC peptides were present whether these linear DNA fragments contained three, two, or one methylated *Pvu*II sites.

INTRODUCTION

Restriction-modification systems. Restriction systems function as the bacterial equivalent of our immune system. Through these systems, bacteria can molecularly monitor the origin of invading or non-self DNA and determine its fate. All DNA possesses the same four bases, so the cell must have some additional means by which it can determine self from non-self. This is accomplished by means of DNA modification in the form of methylation of nucleotides within specific sequences. Foreign DNA detected by the bacterium is inactivated by endonuclease cleavage (57).

Restriction-modification systems are common among bacteria from all ecological niches and taxonomic groups (34). Over 10,000 bacterial strains have been examined and approximately one-quarter have been found to contain sequence specific endonucleases (34, 37). The fact that higher numbers were not seen to possess restriction endonucleases may not be because they are absent, but because there is some unforeseen variable, such as abnormal reaction conditions, unusual target sequences, infrequent cleavage, excessive exonuclease activity, etc.

In addition to restriction endonuclease activity, these restriction-modification systems possess DNA methylase activity that transfers methyl groups from S-adenosyl-methionine (AdoMet) to adenines or cytosines at a specific site. Once methylated, these sites are now protected from the restriction endonuclease activity (81). There are exceptions where the specific endonuclease requires a methylated site for cleavage, such as *Dpn*I.

Restriction-modification systems have been classified into four types based on cofactor requirements, structure, and restriction and modification site specificity. There are two types of ATP-requiring enzymes known as Type I and Type III. Both require ATP and Mg^{2+} for restriction, and both consist of a single multifunctional enzyme. The Type I restriction activity also requires AdoMet, which is not required but does enhance the restriction by Type III systems. Type II systems require AdoMet only for methylation and consist of a separate endonuclease and methylase. The Type IIs systems are quite similar to the Type II systems except that they possess different types of recognition sequences and cleave DNA differently.

Although modification is always sequence specific, some restriction endonucleases cut distal to the methylase recognition site. Type II restriction usually occurs within the methylation recognition site or the methylation/endonuclease site, while Type IIs systems cleave within 20 base pairs (bp) on one side of the recognition site. Type III systems cleave 24 - 26-bp to the 3' side of the host specific site and the Type I restriction system's cleavage site is from several hundred to $>$1000-bp away from the recognition site and is seemingly random in nature.

Type I restriction systems. To date, all Type I restriction systems have been found in the Enterobacteriaceae, such as *Escherichia coli* B and K, *Salmonella typhimurium*, and *Citrobacter freundii*. Fewer than a dozen natural Type I restriction systems have been discovered. Because no definitive screening method exists there may be many more. Throughout these species, this restriction system has repeatedly shown to map at similar or even identical positions on their respective chromosomes (98.5 minutes on the standard *E. coli* map) and have been shown to

be allelic (5, 15, 19, 22). The Type I restriction systems are the most complex of the four types. They have complex cofactor requirements, cut DNA thousands of base pairs from the modification sequence, and are made up of three subunits. The Type I restriction system found in *E. coli* K12 is termed *hsd* for *h*ost *s*pecificity of *D*NA. The genes for this system were designated *hsd* R, M, and S. These three genes occur in this order and are transcribed in the same direction (69). Although this is true, two different promoters have been identified. One promoter is responsible for *hsd* R and the other for *hsd* M and S. This enables synthesis of a methylase composed of *hsd* M and S gene products without the accompanying restriction endonuclease. The R subunit is responsible for restriction, the M subunit for methylation, and the S or specificity subunit for recognizing the target site of DNA. When the S subunit has recognized a target site, the enzyme's binding to DNA may be succeeded either by restriction or modification, which are mutually exclusive events. Which action occurs depends on the state of the target site when bound by the restriction system. If the target site is fully methylated, the enzyme can bind but no further action occurs. If the target is hemimethylated, the enzyme methylates the unmethylated strand. If the target sequence is unmethylated, the enzyme binds tightly in the presence of ATP, and following DNA translocation, cleavage occurs some distance from the recognition site (8, 87). During DNA translocation, the restriction complex remains tightly bound to the recognition sequence and the DNA loops past this complex in a process dependent upon ATP hydrolysis (7, 27, 84, 86). Cleavage is thought to occur when two recognition complexes collide and is a two step event where one strand is cut and then the other

strand is cut nearby (47). Recognition complex collision is able to occur because DNA translocation is thought to occur in both directions (76). Support for this model was obtained by observations via electron microscopy of DNA loops in the presence of this enzyme (84). The fact that small circular DNAs are readily cut, while linear DNA is poorly cut, would seem to support this model also. What selective advantage this cutting at indeterminate distances from the recognition site confers is unclear, but this may serve some role in general recombination (56).

The recognition sites for most Type I enzymes are bipartite consisting of a specific sequence of three base pairs separated by several non-specific nucleotides from a sequence of four base pairs (84). The separation of the two specific sequences is such that both lie on the same face of the DNA helix. These two sequences possess adenine residues that are methylated on opposite strands when modification occurs.

Type III restriction systems. The Type III restriction system is coded for by the *E. coli* prophage P1, the plasmid P15, *Haemophilus influenzae* of serotype R_f, and by *Salmonella typhimurium*. Each of these enzymes consists of two types of subunits. The MS subunit alone acts as a site specific methyltransferase, while as a heterodimer with the R subunit, it acts as an endonuclease as well. Each of these subunits is coded for by its own gene with its own promoter.

The reaction mechanisms of the Type III restrictions systems are very similar. Each requires, but does not hydrolyze, ATP. Non-hydrolyzable ATP analogs can substitute, albeit poorly, for ATP in the restriction reaction. Both ATP and AdoMet have been shown to serve as allosteric effectors of the enzymes in the

cleavage reaction (85). AdoMet is a restriction stimulant but is not required. The presence of both cofactors (ATP and Mg^{2+}), allows Type III enzymes to act as methylases as well as endonucleases, meaning that these activities may act simultaneously as competing reactions. Once bound to its specific site, this subunit competition may result in either DNA methylation or restriction, but it is unclear why this is so. Type III recognition sequences are 5 - 6 nucleotides in length, asymmetric and uninterrupted. Restriction cleavage occurs 24 - 26-bp to the 3' side of the recognition sequence. This may be because the restriction subunit contacts the DNA at this point. Restriction involves staggered cuts, 2 - 4-bp apart. Methylation occurs at adenine residues in the recognition site, but only one adenine on a single strand is methylated. When replication occurs, one new double strand of DNA carries a single methylated adenine, while the other double strand is unmethylated and, conceivably, a target for restriction. However, these endonucleases require the presence of two recognition sites in opposite orientations. Cleavage will occur only if both are unmethylated. Restriction following replication is unlikely as one or the other will remain methylated and preserve the host DNA from restriction (42).

Type II restriction systems. The Type II restriction modification systems are the simplest and most numerous. Over 150 specificities have been identified to date (37, 64). This has been the most highly studied group of restriction-modification enzymes because of their use in modern molecular biology and genetics. The Type II endonuclease requires Mg^{2+}, while the methyltransferase requires AdoMet. Both enzymes are able to act independently. In general,

recognition sequences consist of 4 - 6 bases and have two-fold symmetry. Sequences can be short or long, continuous or interrupted. The palindromic nature of these recognition sites allows one protein to recognize a site on either strand of DNA and to cleave both strands of DNA symmetrically. This cleavage may occur on the 5' or 3' side of the dyad to produce 5' or 3' single-stranded "sticky" termini, while others cleave in the dyad's center to produce "flush" ends. Methylation also occurs symmetrically within the recognition sequence.

Matching methyltransferases and endonucleases virtually always recognize the same target sequence, although they each possess separate target recognition domains. It is thought that recognition of the same target sequence does not necessarily occur when these enzymes first match up, but with time, occurs through evolution (81).

Type IIs restriction systems. The Type IIs restriction-modification systems are similar to the Type II systems. Both have a Mg^{2+}-requiring endonuclease that acts separately from an AdoMet-requiring methyltransferase. The recognition and cleavage sites, however, are quite different. The Type IIs recognition sites are asymmetric and uninterrupted and range in length from four to seven nucleotides. Two-stranded cleavage occurs on only one side of the recognition sequence, and at a defined distance (usually under 20-bp) from the sequence, and is usually staggered by one to four nucleotides (64, 77).

The amino acid sequences of Type IIs methyltransferase are similar to those of the Type II system. The Type IIs methyltransferases probably only differ in that they bind asymmetric nucleotide sequences. Since Type II methyltransferases

recognize palindromic sequences and can bind them in both orientations, they methylate both strands of the sequence. Type IIs methyltransferases can only bind their recognition sequence in one orientation and can, therefore, only modify one strand. Because of this, some Type IIs systems have been seen to possess two methyltransferases, one for each strand. About 30 Type IIs systems have been discovered, but they have not been well characterized. Much is yet to be learned about these complex restriction systems.

Methylation dependent DNA restriction systems. In each of the systems mentioned thus far, the DNA is protected from restriction by methylation. There are systems that specifically recognize and restrict methylated target sites. It is interesting to note that the first restriction systems reported restricted methylated DNA (45). It was noticed that certain bacterial strains restricted T-even phage mutants that, due to mutations in glucosyl transferases (*gt*), lacked glucose covalently bound to hydroxymethylcytosine in their DNA. These *gt* mutations left the hydroxymethylcytosine-containing DNA (30) exposed to restriction activities designated RglA and RglB restriction (*r*estricts *g*lucose*l*ess phage). Rgl restriction was subsequently considered to be an evolutionary relic since at that time no naturally occurring wild-type DNA was known to be sensitive to this system (41, 63). In *E. coli*, the systems Mrr, McrA, and McrBC recognize and restrict DNA methylated at specific sequences.

The Mrr system (modified adenine recognition and restriction) restricts foreign DNA bearing N6-methyladenine (32) and at least one sequence bearing 5-methylcytosine (36). The M.*Sss*I methylase target is the cytosine of all CG

dinucleotides. This creates a similar modification pattern as that seen in eukaryotic DNA and M.*Sss*I modified λ phage have been seen to be sensitive to restriction by the Mrr system. The newfound activity of *mrr* to restrict ^{m}C containing DNA has been designated McrF (37, 41).

The RglA and RglB restriction systems regained importance when it was discovered that they decreased the efficiency of transformation by eukaryotic DNA (52, 60) and the genes for modification methylases (12, 39). This restriction was shown to be sequence specific (60) and modification dependent (52, 60). These restriction systems were renamed Mcr restriction (*m*odified *c*ytosine *r*estriction) (60) systems, and RglA (now McrA) and RglB (now McrBC) were shown to be two separate restriction systems (59). The phrase Rgl restriction has been retained to refer to restriction of T-even phage, while Mcr restriction is used to refer to restriction of all other methylcytosine containing DNA. The *mcrA* gene is located at 25 minutes on the chromosome of *E. coli* K12 on the excisable prophage-like element e14 (59). At least three gene products are encoded in e14 (57), the largest of which is likely McrA. The McrA phenotype restricts the T-even phage *gt* mutants (T2, T4, and T6). The T6 *gt* mutant is most easily used to screen for the McrA system as the McrBC system will not restrict this phage (59). The recognition sequences used to screen for McrA activity were those conferred by M.HpaII (C^{m}CGG) (57) and M.*Sss*I (^{m}CG) (36).

The McrBC restriction system is located at 99 map units on the standard *E. coli* map (59, 68). This has been designated the immigration control region (59) since six different restriction genes are clustered there. Several groups have studied

the McrBC locus and most agree there are two genes producing three gene products. The *mcrB* gene produces 53-kDa ($McrB_L$) and 35-kDa ($McrB_S$) proteins. The smaller McrB polypeptide is produced from an inframe, internal translational start in the *mcrB* gene (67). The $McrB_S$ sequence is identical to $McrB_L$ except for the missing 161 amino acids at the N-terminal end (67). Deletions of the N-terminal coding portion of the *mcrB* gene demonstrate that $McrB_S$ can be expressed independent of the transcription and translation signals for $McrB_L$ (67). The *mcrC* gene produces a single 38-kDa protein (24, 65, 66, 67).

McrBC restriction has been reported to be GTP-dependent and the *mcrB* gene contains a motif similar to the consensus GTP binding site (24, 76). ATP was reported to efficiently inhibit cleavage. GTP analogues with non-hydrolyzable β-γ linkages or mono- or diphosphates do not promote the reaction suggesting a hydrolyzable phosphate bond is necessary (76). McrC has been shown to be basic via sequence analysis (66).

The *mcrB* gene alone is sufficient to restrict DNA methylated by M.*Msp*I (^{m}CCGG) or M.*Sss*I (^{m}CG) (23, 24, 36). To measure this activity, the *mcrB* gene was overexpressed using a plasmid. All other sensitive methylation patterns require the *mcrB* and *mcrC* gene products (23, 24, 66).

Highly purified $McrB_L$ and McrC were used in *in vitro* restriction assays and found to confer the same restriction specificity as extracts prepared from *mcrBC*$^{+}$ cells (76). Using these purified proteins, it was deduced that $McrB_L$ and McrC, but not $McrB_S$ were required for restriction *in vitro*. No activity was seen for $McrB_L$ alone even when DNA modified by M.*Msp*I or M.*Sss*I, which is known to be

restricted by the presence of a functional *mcrB* gene alone, was used as substrate. It is unknown to date, what role $McrB_S$ plays, if any, in restriction.

McrBC activity was initially surveyed using targets which all possessed G^mC or R^mC where R indicates purines (60). The McrBC restriction system is known to restrict mutant phages T2*gt* and T4*gt*, but not T6*gt*. All three have DNA containing hydroxymethylcytosine and it is unclear why T6*gt* is insensitive to restriction. The recognition sequence has recently been proposed to be R^mC (N_{40-80}) R^mC with cleavage occurring at multiple positions on both strands (76). DNA with appropriately spaced methylated bases on only one strand was sufficient for cleavage.

To gain insight into the makeup of the active restriction complex and understand restriction of different DNA substrates, different *in vivo* assays have been used. Rgl restriction is defined as restriction of 5-hydroxymethylcytosine (^{hm5}C) containing DNA (34, 37) and relies on the presence of both genes (*mcrBC*) in the host. This assay is performed by using phage T4*gt*, which possesses ^{hm5}C containing DNA to infect hosts strains to be tested. Greater host restriction is displayed as decreased plaque formation relative to appropriate controls. McrBC restriction is defined as the restriction of DNA containing 5-methylcytosine (^{m5}C) (31, 35). At least 14 patterns are recognized, including the pattern methylated by *Bsu*RI. The restriction assay consists of either infecting test strains with λ bacteriophage which contains ^{m5}C and counting plaques or transforming these hosts with methylase containing plasmids and counting colonies formed. Hosts with this restriction ability are *mcrBC*$^+$. In each case, comparisons are made relative to non-methylated DNA.

McrB* restriction consists of restriction of ^{m5}C-containing DNA also. The *mcrB* gene alone is sufficient to confer this ability and only methylation patterns generated by the *Msp*.I and *Sss*.I methylases are restricted. The McrB* assay is performed either by infection of host strains with λ DNA containing the proper methylation pattern or by transformation of the host with plasmids containing the appropriate methylase gene, such as M.SPR.

In the present study, the role of $McrB_s$ in the McrBC restriction system was explored. The effect of increasing or decreasing the *in vivo* expression of $McrB_s$ was studied. *In vitro* restriction activity and DNA binding were also used to characterize the McrBC system. Based on the findings of this work, a model describing McrBC was proposed.

MATERIALS AND METHODS

Bacteria, phages, plasmids, growth conditions, and media. The bacterial strains, bacteriophage, and plasmid vectors used in this study are listed in Table 1. The *E. coli* strains JM107 (*mcrBC*$^+$) and DH5αMCR (*mcrBC*$^-$) share the common ancestry of MM294. The ER strains are isogenic. The different plasmids selected were used because they had different origins of replication (ori) and were, therefore, compatible in the same host. In some cases, plasmids possessing like ori's were used in the same host. In such cases, the plasmids were constructed in such a way that they possessed differing antibiotic resistance genes. Strains were subcultured in LB broth (48) or on LB 1.5% agar plates. For titering bacteriophage, top agar was made using BBL's trypticase soy broth (0.5%) and 0.8% agar.

Transformation. Plasmids were introduced into *E. coli* strains by either competent cells produced by $CaCl_2$ treatment (44) or by electroporation (25, 26). The former protocol was employed in the McrBC assay that used methylase plasmids and the latter method was used to construct plasmid-containing strains.

McrBC assay using methylated λ phage. McrBC activity refers to the ability of this restriction endonuclease to cleave substrates modified at one of many characterized patterns (52, 59). In this study, the methylation pattern GG^{m}CC of M.*Bsu*RI was used to represent these patterns. Restriction of this pattern requires both $McrB_L$ and McrC proteins (23, 24, 54, 66). A 3.4 kilobase (kb) *Eco*RI fragment containing the M.*Bsu*RI methylase gene from *B. subtilis* R was ligated into *Eco*RI cut pBR322. The resulting plasmid was designated pM.*Bsu*RI10 (39). A *vir* mutant of phage λ was used to infect DH5αMCR (pM.*Bsu*RI10) and the lysate

Table 1. Bacterial strains, bacteriophage, and plasmid vectors

Bacteria, Plasmid Vector or Phage	Relevant Features	Reference
Bacterial strains		
ER1564	*mcrA*, *mcrBC*$^+$, Tet^r, *hsd*, *mrr*	(60)
ER1648	*mcrA*, *mcrBC*, Tet^r, *hsd*, *mrr*	(60)
ER1381	*mcrA*$^+$, *mcrBC*$^+$, Tet^r, *hsd*	(60)
JM107	*mcrA*, *mcrBC*$^+$, *hsd*	(83)
DH5αMCR™	*mcrA*, *mcrBC*, *hsd*, *mrr*	(10)
Bacteriophage		
λ*vir*	*vir*	(71)
T4	wild-type	(30)
T4 *gt*	*gt* (non-glucosylating)	(30)
Plasmids		
pBR322	Ap^r, Tc^r, Col El origin of replication	(14)
pACYC184	Cm^r, Tc^r, p15a ori	(20)
pUC18/pUC19	Ap^r, *rop*, Col E1 ori	(83)
pARV11	Antisense RNA synthesis vector	This study

containing M.*Bsu*RI methylated λ was collected and used to assay McrBC restriction (60). Efficiency of plating (EOP) was determined by dividing the number of plaque forming units (pfu) obtained when infecting strain X with methylated λ by pfu obtained when infecting the permissive host DH5αMCR (pBAB99) with the same phage. For a given strain, restriction was calculated by dividing pfu obtained when infected with non-methylated λ by pfu obtained when infected with methylated λ. Phage λ dilution buffer consisted of 50 mM Tris-HCl (pH 7.8), 0.2M NaCl, and 0.4% $MgSO_4.7H_2O$.

Strains to be assayed were grown to 0.5-1.0 OD_{600} and 300 μl of culture was added to 100 μl of a phage dilution that would yield countable numbers (30-300) of pfu. A dilution of 10^{-8} was used for non-methylated λ, and dilutions of 10^{-6}, 10^{-7}, and 10^{-8} were used for methylated λ. To this phage-cell mixture, 2.5 mls. of 0.8% top agar were added and the entire mix poured onto an L-agar plate. Plaques were counted after overnight incubation. Methylated λ was designated λ.*Bsu*RI and non-methylated λ was designated λ.0.

McrBC assay using methylase plasmids. Transformation of *E. coli* strains with a plasmid encoding the M.*Bsu*RI methylase provides a highly sensitive assay to analyze weakly restricting strains. A 2.0-kb *Cla*I-*Bam*HI fragment was removed from pM.*Bsu*RI10 and ligated into *Cla*I-*Bam*HI-digested pACYC184 and designated pM.*Bsu*RI20. Plasmid DNA was collected using the Birnboim procedure (9) and further purified using the CsCl-ethidium bromide density gradient method (46). Host strains to be tested for McrBC activity were transformed (67) with 0.1 μg of either pM.*Bsu*RI10 or pM.*Bsu*RI20. As compared to the negative or non-restricting strain

DH5αMCR (pBAB99), strains possessing higher restriction levels displayed lower transformation frequencies. Transformation efficiency (number of colony forming units [cfu] per 0.1 μg plasmid DNA) was determined by comparison of potentially restricting host strains with the McrBC-strain DH5αMCR (pBAB99). Restriction was determined by dividing the number of cfu obtained when a given host was transformed with non-methylated plasmid by the number of cfu obtained when transformed with methylated plasmid.

McrB* assay using methylated λ phage. McrB* is defined as restriction observed with the expression of the *mcrB* gene alone and is differentiated from restriction requiring *mcrBC* expression (defined above). A 4.4-kb *Eco*RI fragment from *B. subtilis* R lysogenized with SPR phage was ligated to *Eco*RI-digested pBR322, and the resulting construct was designated pM.SPR10. Again, λ was used to infect DH5αMCR (pM.SPR10) and the lysate containing phage methylated at ^{m}CCGG and GG^{m}CC sites was collected. These methylated λ phage were designated λ.SPR. EOP and restriction were calculated as described in the McrBC assay using methylated λ phage. McrB* activity is observed with the *mcrB* gene alone and can be assayed with DNA modified at the methylation site ^{m}CCGG.

McrB* assay using methylase plasmids. This assay was used as a more sensitive means of measuring McrB* restriction. A 2.8-kb *Cla*I-*Bgl*I fragment from pM.SPR10 was ligated to *Cla*I-*Bam*HI-digested pACYC184, and the resulting recombinant plasmid was designated pM.SPR20. Either this construct or pM.SPR10 were used to transform potentially restricting strains, and restriction levels were

measured by comparing the transformation efficiency to that obtained for the *mcrBC*$^-$ host DH5αMCR (pBAB99).

RglB assay. This assay measures a host strain's ability to restrict non-glucosylated T4 phage DNA (T4*gt*) and was performed as previously described (66). EOP was determined by comparing the number of pfu obtained when infecting a potentially restricting host with T4*gt* to the pfu obtained when infecting the McrBC- strain DH5αMCR (pBAB99). All phage dilutions were made in LB broth. Restriction was calculated for a given strain by dividing pfu obtained due to T4 infection by pfu obtained due to T4*gt* infection.

Antisense RNA-producing plasmid constructs. All antisense RNA producing constructs were derived from pARV11 which combines high level transcription initiation from the *B. subtilis* phage SP82 early gene promoter Sau3A253 (1) with the RNA-stabilizing properties of the *B. thuringiensis cry*A gene terminator (79). pARV11 contains a polylinker site into which small DNA fragments containing the proposed ribosomal binding site (RBS) and initial coding region for each of the three peptides were inserted in the antisense orientation. This results in production of RNA that is stable and in relatively high copy number compared to the single chromosomal copy to be targeted. Antisense RNA1 (ASR1) was designed to block the RBS for $McrB_L$, ASR2 the RBS for $McrB_S$, and ASR3 the RBS for the 38-kDa McrC polypeptide.

pASR1 consists of a *Ssp*I-*Hind*III fragment (nucleotide 73 - 191) (24) ligated into pARV11 that has been digested with *Hind*III and *Hinc*II. pASR2 is made by ligation of an *Ssp*I fragment (nucleotide 470 - 606) (24) into *Hinc*II digested

pARV11. pASR3 was constructed by ligation of an *Eco*RV fragment (nucleotide 1314 - 1518) (24) into *Hinc*II digested pARV11. The orientation of each insert was confirmed by restriction analysis using insert restriction sites located asymmetrically.

Plate method of lawn formation to assay antisense-containing strains. The given strain was grown overnight at 37° C on an L-agar plate which contained the appropriate antibiotic. Cells scraped from the agar surface were suspended in broth to a density of approximately 2.0 OD_{600} units. The cell suspensions were used to create lawns for McrBC assay using phage λ.

SOS induction. The induction of the SOS system was measured with a *sulA* - *lacZ* gene fusion. The plasmid containing the transcriptional fusion pRGC13 was constructed by inserting the *lacZ* gene (via MudI1734) (4) behind the *sulA* promoter (72). The region with the *sulA* - *lacZ* fusion was removed from pRCG13 via a *Bam*HI digestion and ligated to *Bam*HI-digested pACYC184 and called pBAB33.

To test the response of pBAB33 containing strains to SOS induction, mitomycin C was used to induce the SOS response. JM107 (pBAB33) was sampled at approximately 0.3 OD_{600} and 12.5 μl of mitomycin C (final conc. of 0.5 μg/ml) was added to induce the SOS response. Samples were taken 1,2,4, and 6 hours later. Parallel samples were taken from an un-induced culture of the same strain. From these samples, β-galactosidase was assayed as previously described (48) and an induction curve was produced. Restriction-positive strains containing the constructs pBAB33, pBAB33 plus pASR2, or pBAB33 plus pBR322 were grown overnight and, from these, 1 ml was used to inoculate flasks containing 50 ml of L broth (48) with the appropriate antibiotic added. These flasks were shaken in a 37°

C shaker-incubator. β-galactosidase levels in a strain possessing pBAB33 and pASR2 were assayed in cultures with an OD_{600} of approximately 0.5 and 2.25 and compared with this induction curve.

Construction of plasmids for overexpression of McrBC peptides. Construction of pRAB14d (65), pRAB16 (66), and pRAB17 (67) has been explained previously. Another construct used to overproduce the *mcrB* gene products was designated pBAB51. It was constructed by ligating an *Eco*RI-*Nsi*I fragment of pRAB12 (66) into *Eco*RI-*Pst*I digested pUC18 (83). The construct designated pBAB34 was made by removal of the *Nsi*I fragment of pRAB14d (65). This *Nsi*I deletion effectively removes the *mcrC* gene. A 2.75-kb *Eco*RI-*Sca*I fragment from pRAB13 (65, 66) was ligated to *Eco*RI-*Sca*I digested pACYC184 and designated pBAB43. This insert includes *mcrB* only. A 1.6-kb *Eco*RI-*Pst*I fragment from pRAB16 (66) was ligated to *Eco*RI-*Pst*I digested pBR322 and designated pBAB56. This insert includes *mcrC* only and, as this gene lacks its own promoter, it was necessary to provide one in order to see *mcrC* expression. A 301-bp *Eco*RI fragment possessing an early gene promoter from *B. subtilis* phage SP82 was ligated to *Eco*RI-digested pBAB56 and designated pBAB56a.

Plasmids expressing truncated $McrB_S$. A series of plasmids were constructed that contained sequentially greater truncations of the 897-bp $mcrB_S$ gene. Plasmid pBAB70 was constructed by shortening the $mcrB_S$ ORF by 84 nucleotides (leaving 813 nucleotides remaining) through digestion of pRAB14d (65) with *Eco*RV. This digestion removed a 1.4-kb *Eco*RV fragment and the remaining 5.5-kb fragment

was re-ligated to itself. The presumptive product produced from this construct was designated McrB$_S$271.

Plasmid pBAB71 lacks 354 of 897 nucleotides from the *mcrB$_S$* ORF (leaving 543 nucleotides remaining). The presumptive product produced from this construct was designated McrB$_S$181. A *Hind*III deletion was performed on pRAB11 (68), a 0.32-kb fragment removed and the remaining 3.55-kb fragment ligated to itself.

Plasmid pBAB74 lacks 702 nucleotides of the *mcrB$_S$* ORF (leaving 195 nucleotides remaining). The presumptive product produced from this construct was designated McrB$_S$65. A 502-bp *Hind*III-*Bst*UI fragment from pRAB14d (65) was ligated to *Hind*III-*Hinc*II digested pUC19.

The plasmid pASR2 (Materials and Methods, Antisense RNA producing plasmid constructs) was digested with *Bam*HI, and the 144-bp *Bam*HI fragment was ligated into *Bam*HI digested pUC19 and designated pBAB75. This insert consists of the 134-bp *Ssp*I fragment of *mcrB* and lacks 792 nucleotides of the *mcrB$_S$* ORF (leaving 105 nucleotides remaining). The presumptive protein product was designated McrB$_S$35.

McrBC-DNA UV cross linking analysis. To analyze the interaction between the McrBC proteins and DNA, UV photo-cross linking was used to detect protein binding to labeled DNA fragments. Cell extracts from strains producing the various proteins of *mcrBC* were tested for DNA binding. To prepare cell extracts, strains were grown overnight, pelleted, and re-suspended in a binding buffer that consisted of the following: 20 mM HEPES, 2 mM EDTA (pH 8.0), 6 mM Tris-HCl (pH 7.9), 84 mM KCl, and 5% glycerol. Dithiothreitol (DTT) (1 mM final conc.) and

phenylmethylsulfonyl floride (PMSF) (50 μg/ml final concentration) were added to the re-suspended pellet. These cells were lysed by sonication using four, ten-second pulses with the microtip of an ultrasonic processor (Model W-220, Heat System-Ultrasonics, Inc.).

Protein concentration was determined for cell free extracts by using the BIO RAD Protein assay based on the Bradford technique (17) according to the manufacturer's instructions. The binding reaction was set up as follows: 10,000 cpm DNA probe, 0.5 μg poly(dIdC)-poly(dIdC), 1 mM GTP, 300 μg/ml of bovine serum albumin (BSA), and 15 μg protein from crude extract. Actual binding reaction conditions were as follows: 12 mM HEPES (pH 7.9), 1.2 mM EDTA, 3.6 mM Tris-HCl (pH 7.9), 50 mM KCl, and 10% glycerol with a final reaction volume of 15 to 20 μl. To reach these concentrations, binding buffer may be added to the reaction. Each of the binding reactions was mixed into a microcentrifuge tube and then added onto a cellophane-covered heating block set at 30° C. After five minutes of incubation the binding reactions were irradiated with 254-nm UV light (Model SL 253 mineral light, Ultra-Violet Products Inc., South Pasadena, CA.) for 15 minutes from a distance of 3 cm. These samples were then loaded onto a high ionic strength polyacrylamide gel (21) and run at 150 V for approximately 2.5 hours.

Sequencing of target fragments used for McrBC-DNA UV cross linking analysis. The DNA target fragments used in McrBC-DNA UV cross linking analysis possessed one, two, or three *Pvu*II sites. The DNA fragments possessing two and three sites were constructed from ligation of multiple 37-bp fragments. It became necessary to sequence these DNA fragments to discover the distance between

the multiple *Pvu*II sites as this is information relevant to models currently proposed for restriction site requirements. Sequencing was kindly performed by Dr. Alan Biel using the Circumvent™ kit manufactured by New England Biolabs. Thermal cycle sequencing (2) (asymmetric PCR) using plasmid template was performed according to manufacturer's directions.

Target preparation for UV cross linking. A series of DNA fragments containing 1, 2, or 3 *Pvu*II methylation sites were prepared. The *Pvu*II methylation site was constructed by digesting pUC18 with *Pvu*II followed by ligation to delete a 322-bp fragment which included the polylinker cloning sites. The plasmid was designated pBAB99. DNA containing this regenerated *Pvu*II site was cut out with *Hae*III to yield a 37-bp blunt ended fragment which was ligated into SmaI digested pUC18. A plasmid containing one copy of the 37-bp fragment was designated pBAB63, while two and three copies of this fragment were present in pBAB63.11 and pBAB63.1, respectively. The constructs were digested with *Eco*RI-*Bam*HI to remove the entire target insert for end labeling. The *Pvu*II site in the pBAB61 *Eco*RI-*Bam*HI fragment was at least 70-bp from either fragment end. The *Pvu*II site in the same fragment of pBAB62 was 22-bp from one fragment end.

To place the *Pvu*II methylation site on a longer DNA fragment, the plasmid pBAB99 was digested with *Bst*UI, and a 223-bp fragment containing the *Pvu*II site was ligated into *Sma*I-digested pUC18. The resulting construct was designated pBAB62. A second *Pvu*II methylation site was cloned for analysis. Plasmid pBAB61 contains the *Pvu*II methylase site from pBR322. The plasmid pBR322 was digested with *Hae*III and *Msp*I, and the 170-base pair insert containing the *Pvu*II site

was ligated into *Acc*I-*Sma*I-digested pUC18. The target fragments of both pBAB61 and pBAB62 could be removed by *Eco*RI-*Bam*HI for end labeling.

A host strain containing the *Pvu*II methylase gene was transformed with pBAB61, pBAB62, pBAB63.1, or pBAB63.11. All four plasmids were then purified by the procedure of Birnboim (9) and further purified by CsCl-ethidium bromide density gradient centrifugation (46) procedures. Each of these constructs was shown to be methylated by the inability of *Pvu*II to cleave at its sites. Methylated target fragments were generated by digestion with *Eco*RI-*Bam*HI, and DNA fragments were end labeled using [α-^{32}P]dATP (NEN, Dupont) and the Klenow fragment of DNA Polymerase I (Gibco, BRL) according to manufacturer's conditions.

Antibody purification and Western blotting. Polyclonal antibodies against the *mcrB* gene products were produced in rabbits using purified $McrB_L$ and $McrB_S$ (88). This crude antiserum was a kind gift from Dr. H. Douglas Braymer. The polyclonal McrB antibodies were purified using Pharmacia's Protein G Sepharose 4 Fast Flow column with Bio Rad's Econo system. Standard binding (PBS), elution (glycine-HCl, pH 2.7), and neutralizing (BBS) buffers were used (21). The flow rate for antibody binding was 0.5 ml/min. and for elution was 2.7 ml/min. Eluant was collected directly into dialysis tubing, dialyzed against BBS, and then concentrated with an Amicon filter system. The resulting concentrate was allowed to incubate for 30 minutes at 56° C to inactive complement and stored in 0.02% azide at -20° C.

To eliminate background from polyclonal antibodies specific for other *E. coli* proteins (other than McrB), a pretreatment was required. An *E. coli mcrBC* deletion

strain was grown to 0.6 OD_{600} and three, ten ml alloquots were pelleted and suspended in 1% azide PBS for 3 to 4 hours. Antibody was then added to azide-killed cells, suspended to a final dilution of 1/100, and allowed to bind at room temperature for 30 minutes. Cells were pelleted, the supernatant added to a new cell pellet of azide killed cells, and again allowed to bind for 30 minutes. This was repeated once more and supernatant was collected. Sufficient cell free extract from a McrBC deletion strain was added to the supernatant to a protein concentration of 1 mg/ml. Addition of this protein extract yielded a primary antibody final dilution of 1/1,200. The secondary antibody dilution was 1/7,500, and the strepavadin alkaline phosphatase 1/10,000. The blotting detection kit for rabbit antibodies from Amersham was used and all manufacturer's parameters were used, except the previous dilutions listed. The primary antibody was allowed to bind overnight.

Western blots were performed using standard procedures (70). Strains to be used in this assay were pelleted and re-suspended in TEN buffer (50 mM:1 mM:50 mM). PMSF (50 μg/ml final conc.) and DTT (1 mM final conc.) were added to the cell suspension. Cells were lysed by sonication (as described previously) and 40 μg protein extract was loaded onto a Bio Rad 4% - 20% Mini-PROTEAN II Ready gel. The gel was run at 200V for approximately 1 hour using a Bio Rad Mini-PROTEAN II cell. Electroblotting was performed using Bio Rad's Mini Trans-Blot cell at 25 milliamps for 1 hour.

Digestions with McrBC crude extracts. Cell free extracts were used to examine the restriction ability of various strains as well as the necessity of various cofactors. This assay was also performed as an *in vitro* verification that the

restriction positive strains were restricting DNA and that this restriction was dependent on site methylation. Three mls. of an overnight culture were pelleted and suspended in 400 μl of 50 mM Tris-HCl, 1 mM EOTA and 50 mM NaCl (TEN) and PMSF added (final conc. 50 μg/ml). Cells were lysed using sonication (as described previously).

Digestion substrates were pM.*Bsu*RI20 and pNM.*Bsu*RI20. The plasmid pNM.*Bsu*RI20 was produced by transforming ER1381 (*mcrA*$^+$, *mcrBC*$^+$) with pM.*Bsu*RI20. Plasmids were purified from colonies produced from this transformation and digested with *Hae*III as a means to test for any pM.*Bsu*RI20 that had lost their methylation ability. *Hae*III cleavage was taken as evidence that the methylase gene in these constructs was inactive and that these *Hae*III sites were *n*on-*m*ethylated. Upon linearization of pNM.*Bsu*RI20, and comparison with linearized pM.*Bsu*RI20, it was noted that the former appeared slightly larger using agarose gel electrophoresis. This was taken as evidence that an insertion had inactivated the methylase gene.

Digestions were performed using approximately 0.1 μg substrate DNA, 10-20 μl crude extract (30-50 μg protein), 1X React 2 enzyme buffer and different concentrations of GTP or ATP. Digestions were allowed to incubate for one hour at 37° C.

RESULTS

Evidence for subunit interaction. Three different gene products are produced from the *mcrBC* region. The *mcrB* gene produces peptides of 53-kDa ($McrB_L$) and 35-kDa ($McrB_S$). The *mcrC* gene produces a 38-kDa peptide. To examine the relationship between subunit abundance and McrBC activity, these different gene products were over-expressed using various subclones of the *mcrBC* region (Fig. 1). The recombinant plasmid pRAB17 (67) was used to express $McrB_S$ while the plasmid pBAB51 expressed $McrB_L$ and $McrB_S$. The plasmid pRAB16 expressed McrC (66), while pRAB14d (65) expressed $McrB_S$ and McrC. Overproduction of authentic $McrB_L$ alone was not possible as $McrB_S$ is produced from an ORF that is located within the *mcrB* gene. $McrB_S$ is virtually identical to $McrB_L$ except that it lacks 35% of $McrB_L$ from the N-terminal end (67). This means that $McrB_L$ was not produced without synthesis of $McrB_S$ as well.

The *mcrBC*$^+$ host, JM107, was transformed with the various subclones and changes in restriction levels were measured. Restriction was measured as McrBC, McrB*, and Rgl activities using specifically modified substrates. The assays utilized 5-methylcytosine (^{m5}C)-containing-DNA for measure of McrBC or McrB* restriction or 5-hydroxymethylcytosine (^{hm5}C)-containing T4 DNA for measure of Rgl restriction. Phage lambda and plasmids used in the McrBC assay were methylated by M.*Bsu*RI and carried a methylation pattern identical to that produced by M.*Hae*III methylase (GG^{m}CC). Substrate DNA used in the McrB* assay was methylated by M.SPR and carried the same pattern (GG^{m}CC) plus a pattern identical to that produced by M.*Msp*I (^{m}CCGG). Changes in restriction levels were determined by

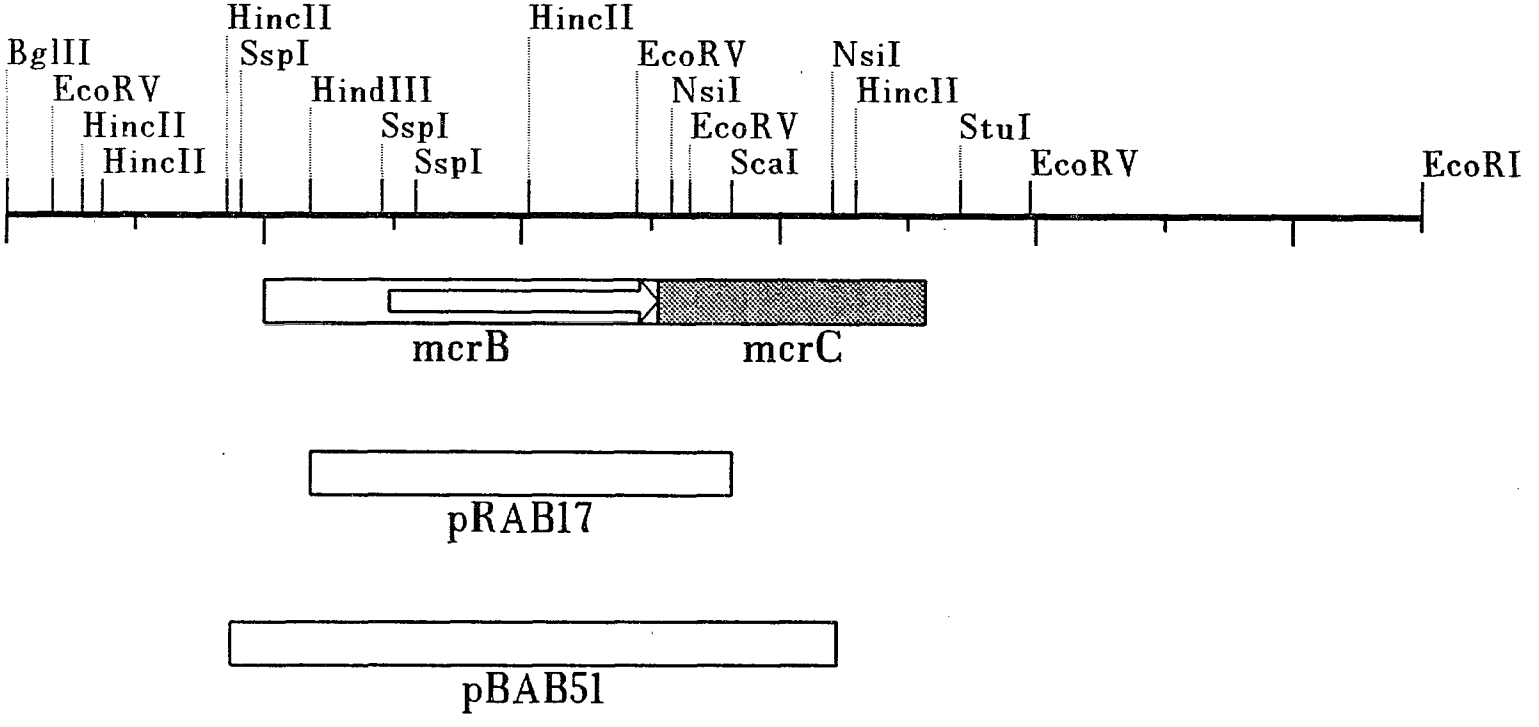


Fig. 1 - Constructs used to overexpress $McrB_L$ and $McrB_S$ in a $McrBC^+$ background. The *mcrB* gene products were overexpressed in a $McrBC^+$ background using pUC8 as the vector for cloning of the above inserts.

comparison of restriction by JM107 containing the relevant subclone, to JM107 (pBAB99) as a positive control and DH5αMCR (pBAB99) as a negative control. Plasmid pBAB99 is a pUC18 derivative with the α-complementation region deleted. Restriction of non-glucosylated bacteriophage T4*gt* DNA was compared to that observed with wild-type bacteriophage T4 DNA.

Wild-type McrBC restriction observed with JM107 (pBAB99) was 43-fold greater than that of the negative control, DH5αMCR (pBAB99). When $McrB_S$ was overexpressed using JM107 (pRAB17), restriction decreased to 2.8-fold (Table 2). This loss of restriction observed when $McrB_S$ was overexpressed was also detected with the Rgl assay. Wild-type Rgl restriction was eliminated with overexpression of $McrB_S$. This was the initial indication that $McrB_S$ affects or modulates the activity of McrBC restriction *in vivo*.

To verify that $McrB_S$ was being overproduced by pRAB17, a Western blot (70) was performed and the relative intensity of band staining compared by densitometry (Fig. 2). Although the effect of $McrB_S$ overexpression on McrBC restriction was examined using JM107, this host strain could not be used for Western blotting. Western blot analysis is not sensitive enough to reproducibly detect wild-type levels of $McrB_S$. Since it was impossible to compare the levels of $McrB_S$ in JM107 to the overexpressed levels observed with pRAB17, the levels of $McrB_S$ produced from two plasmid constructs were compared. To obtain detectable $McrB_S$ levels, pBAB43 was used to express *mcrB* (Fig. 3). This plasmid is a derivative of the vector pACYC184 with approximately 20 copies/chromosome (20). In DH5αMCR (pBAB43, pBAB99), the $McrB_L/McrB_S$ ratio was 13/1. With the strain

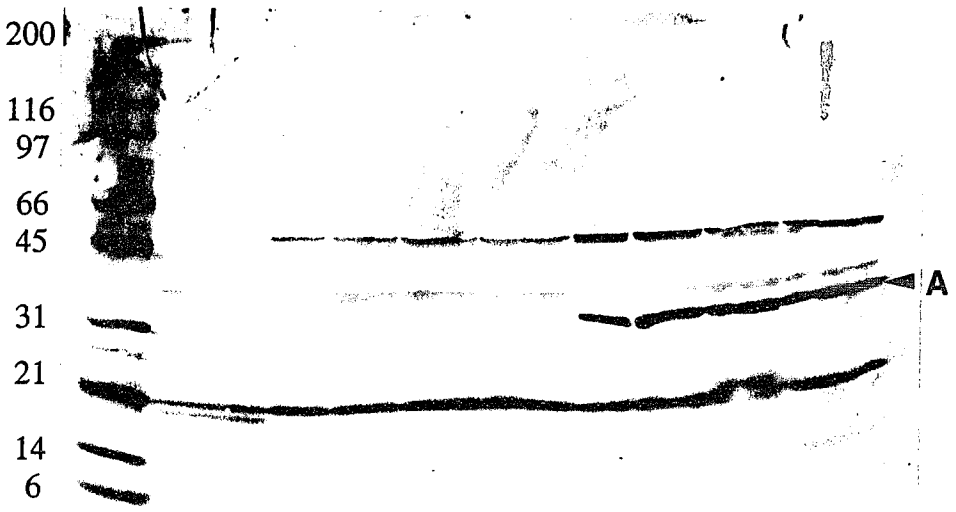
Table 2. The effect of subunit overexpression on McrBC restriction

McrB Restriction	Efficiency of Plating (EOP)[a]		Restriction[b]
	λ.0	λ.*Bsu*RI	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pBAB99)	1.3	3.0×10^{-2}	43
JM107 (pRAB16)	0.9	3.0×10^{-1}	3.0
JM107 (pRAB17)	1.1	3.9×10^{-1}	2.8
JM107 (pBAB51)	1.2	2.0×10^{-2}	60
JM107 (pRAB14d)	1.0	2.0×10^{-2}	50
JM107 (pBAB34)	1.0	2.5×10^{-1}	4.0
	Efficiency of Plating (EOP)[a]		**Restriction[c]**
Rgl Restriction	**T4**	**T4 gt**	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pBAB99)	0.6	4.5×10^{-6}	1.3×10^{5}
JM107 (pRAB16)	0.4	0.8	0.5
JM107 (pRAB17)	0.7	0.5	1.4
JM107 (pBAB51)	0.7	6.2×10^{-7}	1.1×10^{6}
JM107 (pRAB14d)	0.4	2.5×10^{-6}	1.6×10^{5}
JM107 (pBAB34)	0.7	0.2	3.5

a Efficiency of plating is defined as: titer of the phage on strain x / titer of the phage on permissive host DH5αMCR (pBAB99).

b Restriction is defined as: restriction of non-methylated DNA / restriction of methylated DNA.

c Restriction is defined as: restriction of glucosylated DNA / restriction on non-glucosylated DNA.



Lane	1	2	3	4	5	6	7	8	9	10
μg Protein	N/A	70	20	30	50	70	20	30	50	70

Fig. 2 - Western blot analysis for overproduction of $McrB_S$. Lane 1 contains protein standards; numbers represent molecular mass in kDa. Lane 2 contains extract prepared from McrBC$^-$ cells. Lanes 3 - 6 contain extract from DH5αMCR (pBAB43, pBAB99). Lanes 7-10 contain extract from DH5αMCR (pBAB43, pRAB17). Amounts of extract added to each lane is listed. Note that the $McrB_L$ band is designated by an arrow labeled "A".

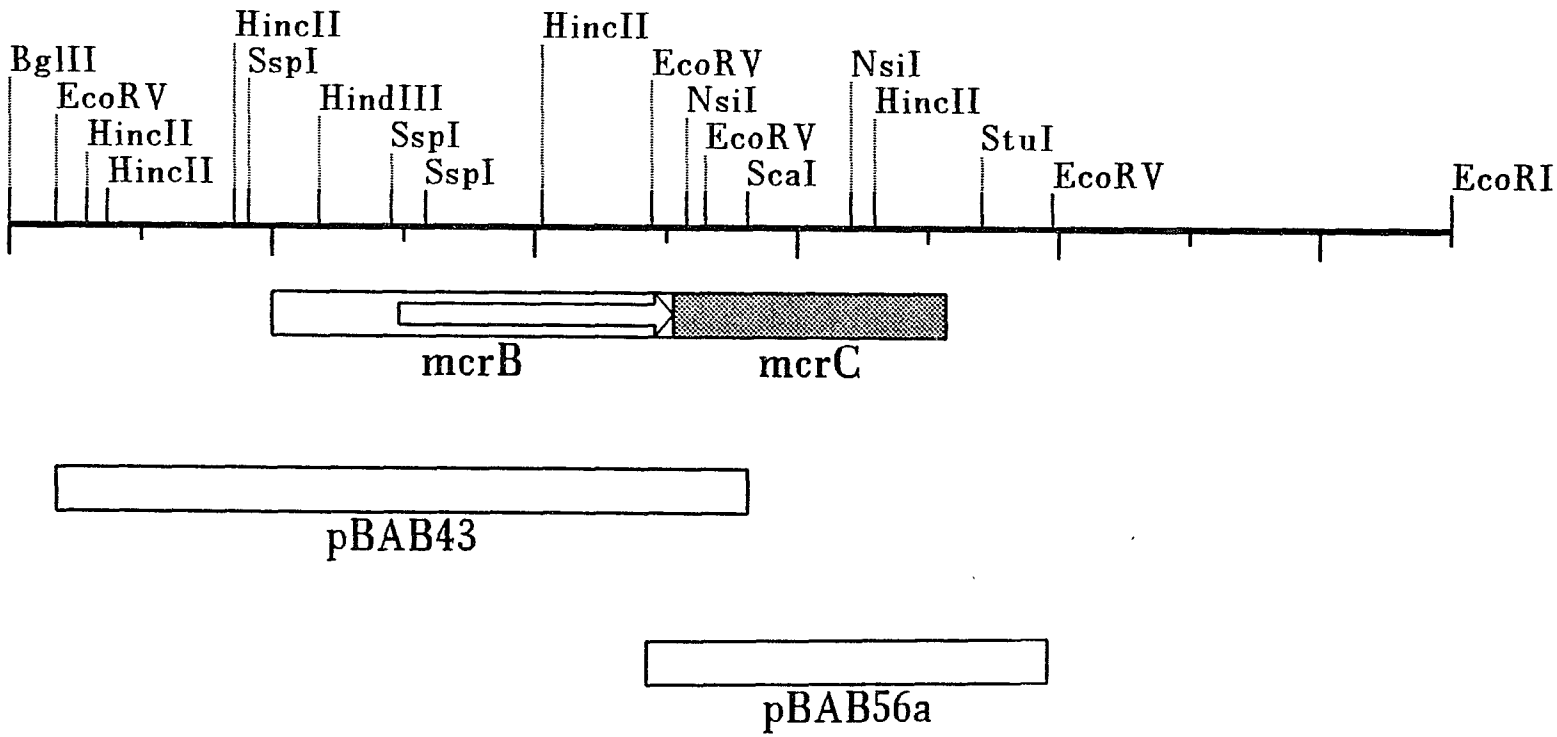


Fig. 3 - Subclones of McrBC region used to express genes individually. Plasmid pBAB43 was constructed with the vector pACYC184 and was used in the McrB* assay. Plasmid pBAB56a was constructed using pBR322 and was used for the "restriction rescue" assay.

DH5αMCR (pBAB43, pRAB17), the ratio changed to 0.8/1. This represents a 16-fold increase in $McrB_S$ levels while the level of $McrB_L$ remains unchanged. It should be noted that the ratio of $McrB_L/McrB_S$ is based on detection with polyclonal antibody and can only be used to compare the relative levels of $McrB_S$ between strains. Since the background levels of $McrB_L$ and $McrB_S$ are provided by a plasmid with a copy number 20-fold greater than the chromosome, determinations of $McrB_S$ overproduction were underestimated by as much as a factor of 20. Estimations of $McrB_S$ overproduction place it at 320 times that of the wild-type levels.

When $McrB_S$ is overproduced, restriction is diminished. Since overproduction of $McrB_S$ did not decrease the levels of $McrB_L$, it is unlikely that $McrB_S$ overproduction results in repression of $McrB_L$. To test if it is the absolute levels of $McrB_S$ or the altered ratio of $McrB_S$ to other components in the restriction system, $McrB_L$ and $McrB_S$ were overproduced with pBAB51. When both $McrB_L$ and $McrB_S$ were overproduced as verified by Western blot using JM107 (pBAB51), there was no loss of restriction (Table 2). In fact, there was a slight but reproducible increase in restriction ability. This suggests that overproduction of both *mcrB* products does not disrupt the subunit ratio necessary for the formation of the active complex. These results suggest that it is the altered proportions of $McrB_S$ to $McrB_L$ that is inhibitory, that $McrB_L$ and $McrB_S$ interact and/or that these two proteins interact with a common protein, which is likely McrC. Both of these hypotheses were tested.

$McrB_L$-$McrB_S$ interaction. Using the McrB* restriction assay, additional evidence was sought that $McrB_L$ and $McrB_S$ interact. Full restriction of λ.SPR as

defined by the positive control JM107 (pBAB99) was 61 fold. The *mcr* deletion strain DH5αMCR was transformed with pBAB43, which encodes the *mcrB* gene, and when infected with λ.SPR, restriction was measured at 45-fold greater than the negative control DH5αMCR (pBAB99). The strain DH5αMCR (pBAB43, pRAB17) displayed 25.6-fold restriction (Table 3). The presence of pRAB17 caused the overproduction of $McrB_S$ and suggested that $McrB_S$ interacted with $McrB_L$ and inhibited the McrB* activity.

As stated previously, pRAB17 caused a 16-fold increase in $McrB_S$ levels in this strain. While not dramatic, the loss of restriction from 45 fold to 26 fold is repeatable. It is thought that this restriction loss is not greater because the *mcrB* to $mcrB_S$ ratio is approximately 1:10, rather than 1:200 as would be the case if a chromosomally encoded $mcrB^+C^-$ host was available. These ratios were calculated using the copy numbers of the plasmid vectors used.

$McrB_S$-McrC interaction. Overexpression of McrC was achieved using JM107 (pRAB16). This resulted in a change from 43-fold McrBC restriction, as seen with JM107 (pBAB99) to 3-fold McrBC restriction (Table 2). Overexpression with pRAB16 also caused complete elimination of Rgl restriction. This suggests that excess McrC disrupts active complex formation, or represses the synthesis of McrB. Western blot analysis detected no effect on synthesis of $McrB_L$ or $McrB_S$ when McrC was overexpressed (data not shown). The possibility that McrC overexpression affected active complex formation was tested.

Overexpression of either $McrB_S$ or McrC dramatically reduced McrBC and Rgl restriction. To test whether these two proteins interact, the host JM107

Table 3. The effect of overexpression of truncated *mcrB*$_S$ genes on McrB* restriction

	Efficiency of Plating (EOP)[a]		Restriction[b]	Efficiency of Plating (EOP)[a]		Restriction[b]
	λ.0	λ.SPR		λ.0	λ.*Bsu*RI	
DH5αMCR (pBAB99)	1.0	1.0	1.0	1.0	1.0	1.0
JM107 (pBAB99)	1.1	$1.8x10^{-2}$	61	0.9	0.02	45
DH5αMCR (pBAB43, pBAB99)	0.9	$2.0x10^{-2}$	45	0.8	1.0	0.8
DH5αMCR (pBAB43, pRAB17)	1.1	$4.3x10^{-2}$	26	0.9	1.0	0.9
DH5αMCR (pBAB43, pBAB70)	0.9	$2.2x10^{-2}$	41	1.0	0.8	1.3
DH5αMCR (pBAB43, pBAB71)	1.2	$2.5x10^{-2}$	46	1.2	1.2	1.0
DH5αMCR (pBAB43, pBAB74)	0.7	$7.0x10^{-3}$	100	0.9	0.7	1.3
DH5αMCR (pBAB43, pBAB75)	0.4	$1.3x10^{-3}$	320	0.25	0.44	0.6

a Efficiency of plating is defined as: titer of the phage on strain x / titer of the phage on permissive host DH5αMCR (pBAB99).

b Restriction is defined as: restriction of non-methylated DNA / restriction of methylated DNA.

(*mcrBC*$^+$) was transformed with pRAB14d (65) which expresses $McrB_S$ and McrC as previously verified by maxi-cell analysis (65). When both $McrB_S$ and McrC proteins were overexpressed together, no loss of restriction was seen. Restriction was at or slightly above that of the positive control, JM107 (pBAB99) (Table 2). We term this "restriction rescue," meaning the loss of restriction caused by overproduction of $McrB_S$ or McrC in JM107 is restored when both are produced together. It was previously shown that pRAB14d does not impart McrBC restriction of *mcrBC*$^-$ hosts (65). To confirm that both peptides expressed from pRAB14d are required for restored activity, pBAB34 was created from pRAB14d (Fig. 4) by deleting the McrC coding region. JM107 was transformed with pBAB34, and McrBC and Rgl assays were performed (Table 2). Full restriction was represented by JM107 (pBAB99) and was seen as Rgl restriction of 1.3×10^5 fold and McrBC restriction of 43 fold above restriction levels of the negative control DH5αMCR (pBAB99) (Table 2). With pBAB34 (Table 3), there is virtually no restriction observed as would be expected with an over-producer of $McrB_S$ in a wild-type host. An Rgl restriction level of 3.5 fold and an McrBC restriction level of 4 fold are comparable to those levels seen with $McrB_S$ overproduction in JM107 (pRAB17) and with the negative control DH5αMCR (pBAB99).

To verify these findings, the wild-type strain (JM107) was transformed with pBAB56a (Fig. 3). This resulted in overexpression of McrC and caused McrBC restriction to drop from 90 fold to 8 fold. When JM107 (pBAB56a) was transformed with pRAB17, which encoded overproduction of $McrB_S$, "restriction rescue" was seen to occur, and full restriction was once again seen. At 128-fold,

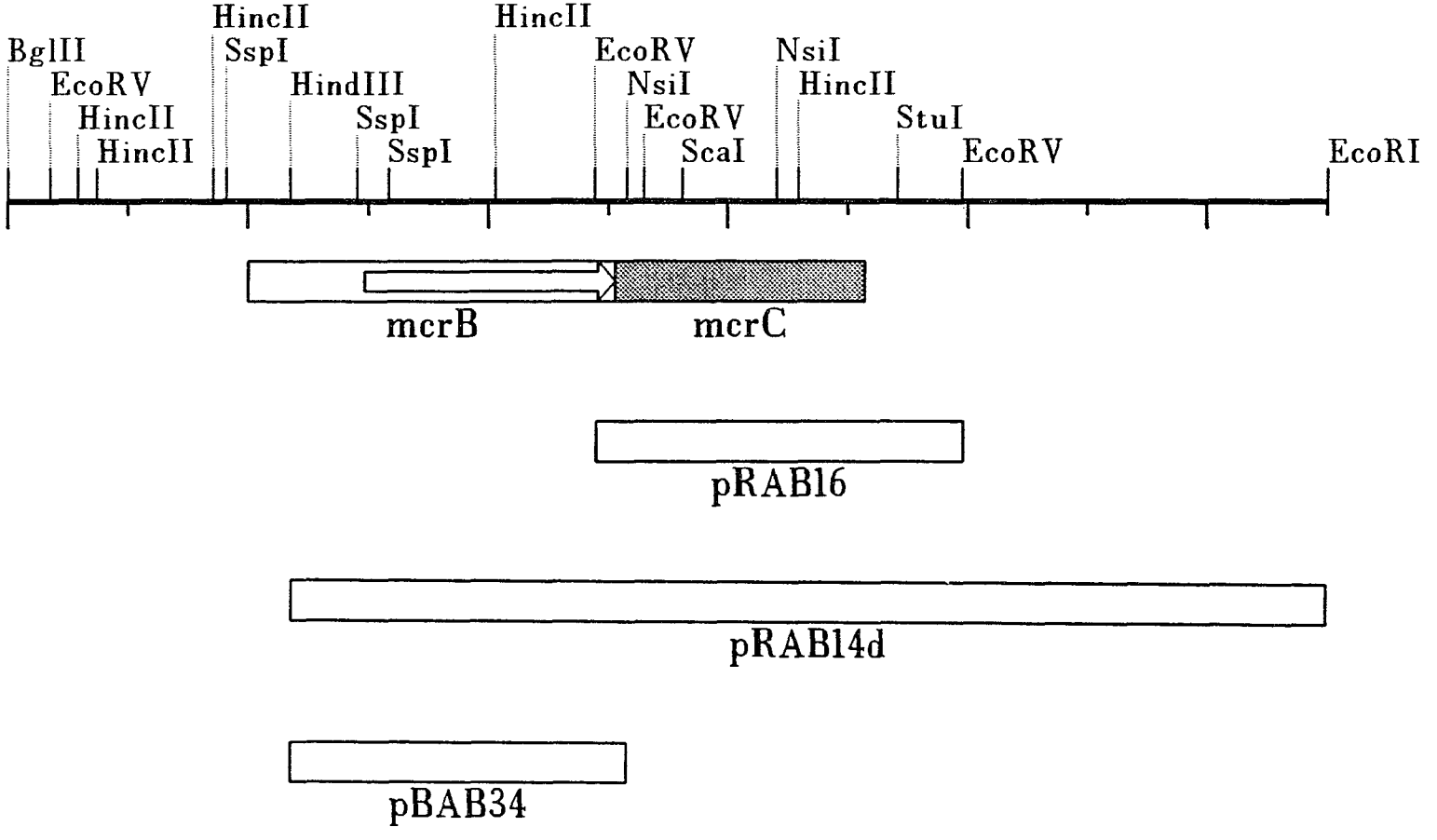


Fig. 4 - Constructs used to test $McrB_S$-McrC interaction. The open boxes represent the fragment of DNA used to construct the listed plasmids. All constructs were made using the vector pUC8.

restriction was observed at, or slightly above, that observed with the positive control, JM107 (pBAB99).

While overexpression of either McrC or $McrB_S$ leads to loss of restriction, simultaneous overexpression of these proteins permits full restriction by chromosomally encoded McrBC. Alone or together McrC and $McrB_S$ cannot impart restriction to DH5αMCR (*mcrB⁻C⁻*). This suggests that McrC and $McrB_S$ interact or complex together to prevent them from disrupting normal McrBC restriction.

Use of antisense RNA to affect McrBC restriction. To decrease the levels of the individual proteins produced from the *mcrBC* operon, antisense RNAs were made using pARV11 as the vector (Fig. 5). These antisense RNAs were made in such a way that they blocked the translational initiation sites of $McrB_L$, $McrB_S$, and McrC (Fig. 6). It should be noted that all changes in EOP levels are made in comparison to the restriction negative control DH5αMCR (pBAB99) and changes in restriction are compared to full restriction as expressed by JM107 (pARV11).

The *mcrBC*$^+$ host JM107 was transformed with the construct designated pASR1 to inhibit synthesis of $McrB_L$. With pASR1 present, McrBC restriction drops from 30 fold to 4.6 fold and Rgl restriction drops from 7.0×10^5 fold to 50 fold (Table 4). This result was entirely expected as $McrB_L$ is the restriction subunit and necessary for restriction of all targets (24, 65, 66). It should be noted that with pASR1 present, JM107 displayed healthy growth, and cells were observed to be normal when viewed with a phase-contrast microscope.

Inhibition of McrC synthesis in JM107 using the construct designated pASR3 resulted in a decrease in McrBC restriction from 30 fold to 4.5 fold and in Rgl

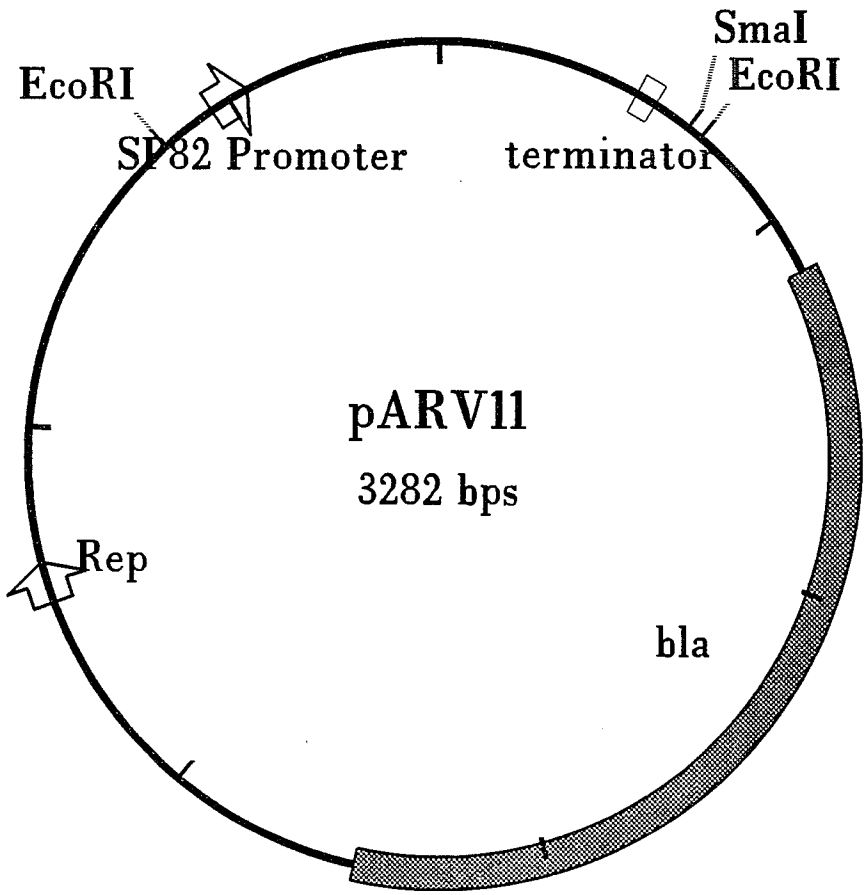


Fig. 5 - Antisense RNA vector. This vector combines high level transcription initiation from the *B. subtilis* phage SP82 early gene promoter *Sau*3A253 with the RNA stabilizing properties of the *B. thuringiensis cry*A gene terminator.

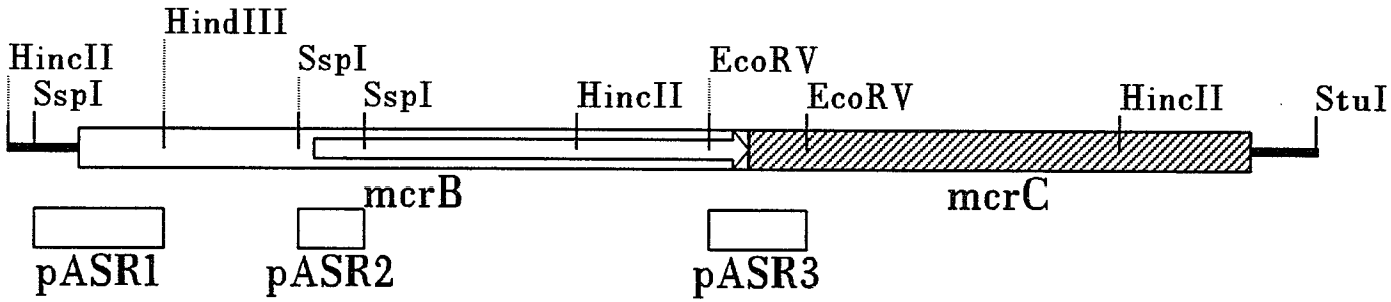


Fig. 6 - Antisense RNA producing constructs. Each of the above DNA fragments was cloned in antisense orientation using the vector pARV11. The resulting RNA produced targeted the ribosome binding sites of the three McrBC protein products.

Table 4. Use of antisense RNA to facilitate underexpression

McrB Restriction	Efficiency of Plating (EOP)[a]		Restriction[b]
	λ.0	λ.*Bsu*RI	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pARVII)	1.5	5×10^{-2}	30
JM107 (pASR1)	1.3	0.3	4.5
JM107 (pASR2)	1.6	0.3	5.9*
JM107 (pASR3)	1.3	0.3	4.5

Rgl Restriction	Efficiency of Plating (EOP)[a]		Restriction[c]
	T4	T4 gt	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pARVII)	1.2	1.7×10^{-6}	7×10^{5}
JM107 (pASR1)	1.5	3.0×10^{-2}	50
JM107 (pASR2)	1.3	3.0×10^{-2}	43*
JM107 (pASR3)	1.1	0.6	2

a Efficiency of plating is defined as: titer of the phage on strain x / titer of the phage on permissive host DH5αMCR (pBAB99).

b Restriction is defined as: restriction of non-methylated DNA / restriction of methylated DNA.

c Restriction is defined as: restriction of glucosylated DNA / restriction on non-glucosylated DNA.

* Although observed restriction levels with JM107 (ASR2) were variable, this restriction level was repeatable using the "plate scraping" method.

restriction from 7 x 10^5 fold to 2 fold (Table 4). Since McrC in conjunction with $McrB_L$ is known to be necessary for restriction of most methylated DNA templets (24, 40, 51, 65) and ^{hm5}C containing DNA (58, 61), lowered amounts of McrC produced expected results of dramatically reduced McrBC and Rgl restriction. It follows that lowered expression of McrC should not affect McrB* restriction as this restriction ability can occur with *mcrB* alone. This in fact proved to be true. In a side-by-side assay, ASR3 was shown to reduce McrBC restriction while not altering McrB* restriction. JM107 (pASR3) was also seen to possess normal growth and appearance when viewed with a phase-contrast microscope.

Plasmid pASR2 was constructed to inhibit synthesis of $McrB_S$. Presence of this construct in JM107 had two immediately evident effects: (1) a slow growing cell that lysed upon serial transfer in broth medium, and (2) variable results of the McrBC and Rgl assays. In early attempts to assay JM107 (pASR2), this construct slightly increased restriction, 2 to 5 fold. These results were not readily repeatable as cells quickly lysed when growing in the incubator shaker. A method was devised to produce bacterial lawns with cells grown on solid media where they were most stable. These cells were infected with bacteriophage T4 strains (Rgl assay) or bacteriophage λ*vir* lysates prepared for the McrBC assay. Under these conditions, ASR2 lowered McrBC restriction from 30 fold to 6 fold, and Rgl restriction from 7 x 10^5 fold to 43 fold. These results again suggested that $McrB_S$ had some significant role in the McrBC restriction system and even in cell stability. When viewed with a phase contrast microscope, JM107 (pASR2) cells appeared as non-

septated, filamentous, and pleomorphic. These growth abnormalities are consistent with SOS induction.

Western blotting (70) and densitometry were used to verify that ASR2 had caused underproduction of $McrB_S$ only (Fig. 7). The antisense RNA experiment utilized JM107 (pASR2), but this strain could not be used for Western blotting as this assay is not sensitive enough to detect chromosomally expressed levels of $McrB_S$. To obtain levels of $McrB_S$ that were measurable, pBAB43 was used to express *mcrB* in the *mcrBC* deletion strain DH5αMCR. This construct utilizes pACYC184, which has a copy number of 20 plasmids per chromosome. In DH5αMCR (pBAB43, pBAB99), the $McrB_L/McrB_S$ ratio was seen to be approximately 4/1. In DH5αMCR (pBAB43, pASR2), the ratio changes to 11/1. This represents an almost 3-fold drop in the amount of $McrB_S$ in the presence of ASR2 while $McrB_L$ levels remain unchanged. While this 3-fold drop in $McrB_S$ demonstrates the ability of ASR2 to inhibit synthesis of this protein, it is a gross underestimate of what would be expected from a chromosomally encoded gene. The levels of $McrB_S$ in JM107 (pASR2) could be as much as 20 fold lower than that determined for DH5αMCR (pBAB43, pASR2).

It was hypothesized that the introduction of ASR2 actually *increased* restriction, but this increase was so dramatic that chromosomal damage occurred. This would trigger the SOS repair system and error prone DNA repair. The ability of pASR2 to induce SOS was examined.

It should be noted that $McrB_L/McrB_S$ ratios stated in this study were only relevant to a particular Western blot. These blots were performed to report a *change*

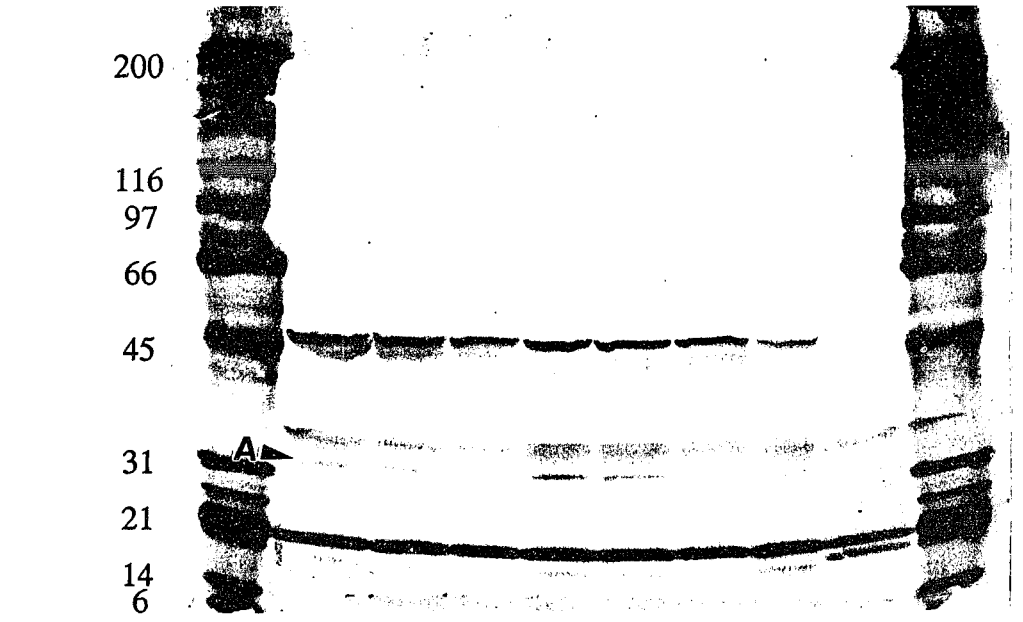


Fig. 7 - Western blot analysis of $McrB_S$ underproduction caused by ASR2. Lanes 2 - 4 contain extract from DH5αMCR (pBAB43, ASR2). Lanes 6 - 8 contain extract from DH5αMCR (pBAB43, pARV11). Lane 8 contains 70 μg from DH5αMCR (pBAB43, pBAB99), and Lane 9 contains extract from DH5αMCR (pBAB99). Extract amounts are listed as μg protein. The arrow labeled "A" marks the position of the $McrB_S$ product band. Molecular weight markers in Lanes 1 and 10 are designated in kDa.

in this ratio only and these numbers should not be taken as relevant to the actual $McrB_L/McrB_S$ ratio found in the cell. Ratios varied from blot to blot as the same polyclonal antibody alliquot was used repeatedly. $McrB_L$ and $McrB_S$ share a common region and antibodies against this shared region had their numbers exhausted before antibodies specific for the unique region found on $McrB_L$. Because of this, early uses of a given antibody dilution yielded a $McrB_L/McrB_S$ ratio closer to one than later uses of the same dilution. After repeated antibody uses, $McrB_S$ appeared in lower amounts relative to $McrB_L$ simply because antibody specific for the unique region of $McrB_L$ was removed from the polyclonal antibody population more slowly.

SOS induction as measured by β-galactosidase activity. To test the hypothesis that the slow growing, filamentous cells observed when JM107 was transformed with pASR2 were due to the SOS response, an SOS inducible reporter plasmid was constructed with a *sulA-lacZ* fusion. This plasmid, pBAB33, places β-galactosidase synthesis under the control of the LexA repressible *sulA* promoter. To test the ability of pBAB33 to respond to SOS induction by DNA damage, *E. coli* strain JM107 (pBAB33) was grown in two separate flasks. Mitomycin C at a final concentration of 0.5 μg/ml was added to one flask to induce SOS while the other flask served as a control. Both cultures were sampled upon time of induction and at 1, 2, 4, and 6 hours post induction. Based on the β-galactosidase specific activity determined with these samples, mitomycin C addition resulted in approximately 2-fold increase in expression of *sulA-lacZ*. The non-induced sample starts at 63 Miller units and levels off at 119 units. The SOS induced sample displayed a beginning

reading of 55 Miller units and a final reading of 203 Miller units. Based on this control, a two-fold increase in β-galactosidase activity in pBAB33-containing strains would be consistent with SOS induction.

When JM107 (pBAB33) was transformed with pASR2, SOS induction was observed. Initial samples taken had an OD_{600} of 0.55 and a β-galactosidase specific activity of 106 Miller units. This level of β-galactosidase was twice that of untreated JM107 (pBAB33) at a similar optical density. With an OD_{600} reading of 2.23, the β-galactosidase specific activity was 198 Miller units, which corresponds with mitomycin-induced JM107(pBAB33) at comparable optical density. For the control JM107 (pBAB33, pBR322) at an OD_{600} of 2.19, β-galactosidase specific activity was measured at 64 Miller units. Since the antisense RNA produced from pASR2 specifically targeted $McrB_S$ synthesis, the SOS response appears related to the underproduction of this protein. This effect was only observed in cells with a functional *mcrBC* operon. This suggests that DNA damage results from McrBC restriction system when adequate levels of $McrB_S$ are not present.

Altered $McrB_S$ proteins. To explore the possible interactions between $McrB_S$ and the two subunits essential for McrBC restriction, $McrB_L$ and McrC, a series of plasmids that expressed altered $McrB_S$ proteins were made. The altered $McrB_S$ proteins were analyzed with respect to three different parameters: (1) effect on McrBC restriction, (2) ability to interact with the McrC protein using the "restriction rescue" assay, and (3) ability to interact with $McrB_L$ and effect McrB* restriction.

(1) A series of plasmid constructs was made that encode $McrB_S$ proteins with sequentially greater truncations at the C-terminal end (Fig. 8). When overexpressed in JM107, each of these truncated *mcrB*$_S$ genes, encodes a fraction of the full $McrB_S$ protein (Table 5). Each plasmid construct was tested for its effect on McrBC restriction using bacteriophage λ modified by the *Bsu*RI methylase. As demonstrated above, when the full length *mcrB*$_S$ gene is overexpressed in JM107, McrBC restriction is significantly inhibited. Specifically, full restriction levels represented by JM107 (pBAB99) are 60 fold above the negative control, while overproduction of $McrB_S$ in JM107 (pRAB17) decreases restriction to only 2.6 fold above the control.

When each of the truncated $McrB_S$ proteins was tested, it was observed that as the length of the C-terminal truncation increased, the ability of the $McrB_S$ protein to inhibit the McrBC restriction decreased. When the last 81 nucleotides of the *mcrB*$_S$ gene were deleted (i.e. representing 27 of the 298 amino acids in $McrB_S$) as in the plasmid construct pBAB70, restriction increased slightly to 7.7 fold above the negative control. A second truncated $McrB_S$ protein produced from pBAB71 contains 181 out of the 298 amino acids in the full length protein. When overexpressed in JM107 restriction was 52 fold, essentially the same as observed with no $McrB_S$ overexpression. This truncation appeared to have eliminated the ability of the $McrB_S$ protein to inhibit McrBC restriction.

More extensive truncations of the *mcrB*$_S$ gene produced some interesting findings. The construct designated JM107 (pBAB74) displayed 120-fold restriction. This level was significantly higher than the positive control JM107 (pBAB99) and

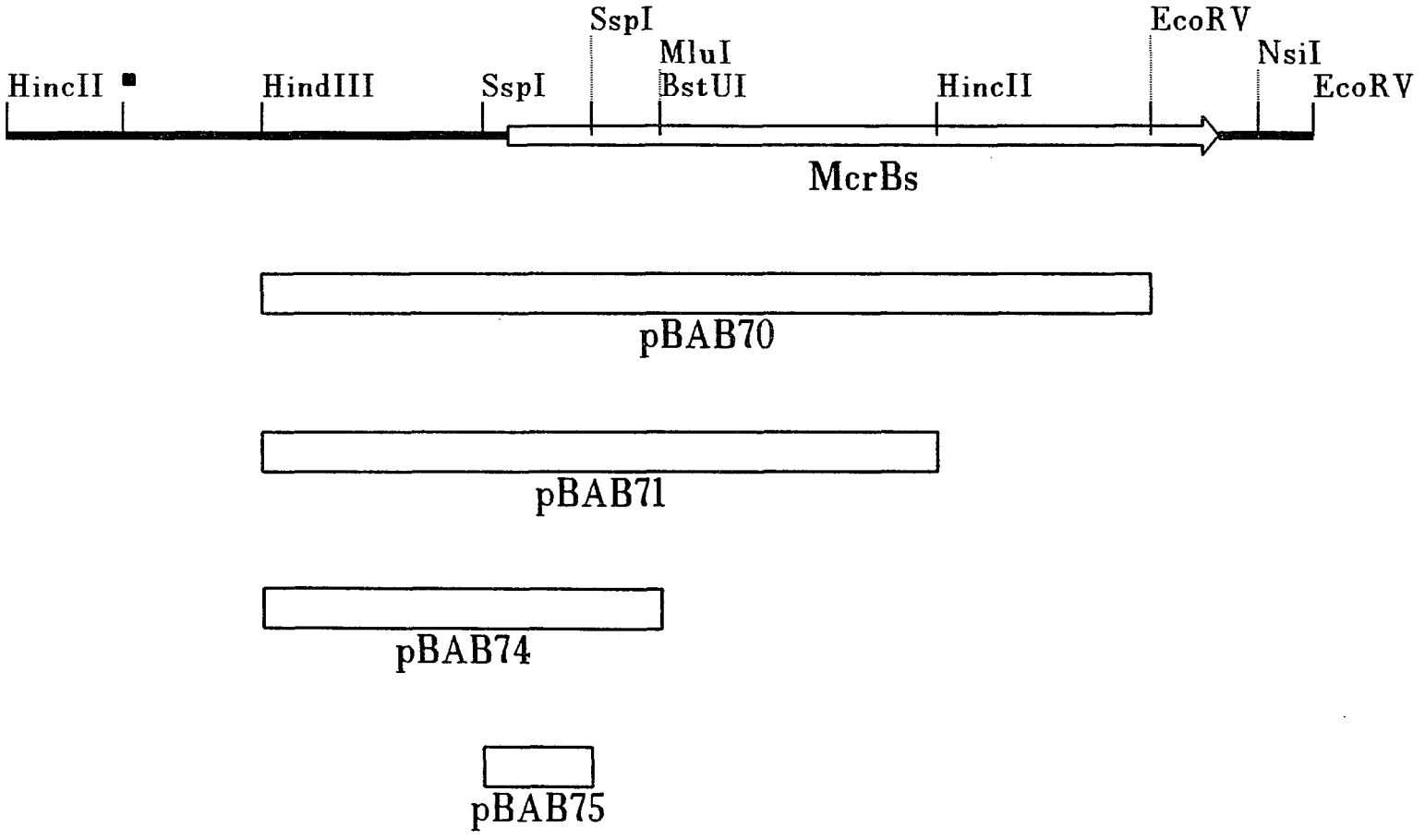


Fig. 8 - Plasmids expressing truncated *mcrB*$_S$ genes. These constructs allow production of truncated McrB$_S$ proteins. The open boxes represent the DNA fragment cloned into a pUC vector to make the listed construct.

Table 5. Predicted length of products from truncated *mcrB*$_S$ genes

Plasmid	Product Name[a]	Total codons in Product
pRAB17	McrB$_S$	298
pBAB70	McrB$_S$271	317
pBAB71	McrB$_S$181	224
pBAB74	McrB$_S$65	67
pBAB75	McrB$_S$35	53

a Presumptive product names are based on the number of McrB$_S$ amino acids remaining out of 298.

was termed "hyper-restriction". This elevated restriction is caused by $McrB_S65$, which consists of 65 of 298 amino acids of $McrB_S$. While overexpression of this truncated $McrB_S$ no longer inhibits McrBC activity, it can still interact with the complex as evidenced by the hyper-restriction. The construct JM107 (pBAB75) contained the smallest segment of *mcrB*$_S$, 105 out of 897-bp, with the predicted peptide, $McrB_S35$, containing only 35 amino acids from $McrB_S$. McrBC restriction was 220 fold greater than the negative control (Table 6). This suggested that $McrB_S35$ maintains the binding ability of $McrB_S$ without the regulatory ability. The elevated levels of McrBC restriction may be due to $McrB_S35$ competing with endogenous $McrB_S$ that is able to inhibit McrBC restriction. None of these plasmids alone were seen to confer any restriction ability to the McrBC$^-$ host DH5αMCR (data not shown). It should be noted that cultures of strains that exhibited hyper-restriction, JM107 (pBAB74) and JM107 (pBAB75), displayed slightly slower generation times and greater numbers of filamentous cells than the untransformed host.

(2) The overexpression of *mcrC* in JM107 resulted in a loss of restriction. The overproduction of $McrB_S$ in concert with McrC allowed "restriction rescue" or recovery of full restriction (Table 7). Full restriction was established by JM107 (pBAB99) at 90 fold greater than the negative control. When transformed with pBAB56a, which encodes *mcrC*, restriction dropped to 7.7 fold. When JM107 (pBAB56a) was transformed with pRAB17, which encodes *mcrB*$_S$, restriction was rescued and 130-fold restriction was seen. The series of plasmids encoding truncated *mcrB*$_S$ genes was then examined to identify regions of $McrB_S$ that interact

Table 6. The effect of overexpression of truncated *mcrB*$_S$ genes on McrBC restriction

	Efficiency of Plating (EOP)[a]		Restriction[b]
	λ.0	λ.*Bsu*RI	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pBAB99)	1.2	2.0×10^{-1}	60
JM107 (pRAB17)	1.0	3.9×10^{-1}	2.6
JM107 (pBAB70)	1.0	1.3×10^{-1}	7.7
JM107 (pBAB71)	7.8×10^{-1}	1.5×10^{-2}	52
JM107 (pBAB74)	1.1	9.0×10^{-3}	120
JM107 (pBAB75)	1.2	5.5×10^{-3}	220

a Efficiency of plating is defined as: titer of the phage on strain x / titer of the phage on permissive host DH5αMCR (pBAB99).

b Restriction is defined as: restriction of non-methylated DNA / restriction of methylated DNA.

Table 7. The study of truncated *mcrB*$_S$ genes using the restriction rescue assay

	Efficiency of Plating (EOP)[a]		Restriction[b]
	λ.0	λ.*Bsu*RI	
DH5αMCR (pBAB99)	1.0	1.0	1.0
JM107 (pBAB99)	0.9	1.0×10^{-2}	90
JM107 (pBAB56a, pBAB99)	1.0	1.3×10^{-1}	7.7
JM107 (pBAB56a, pRAB17)	0.9	7.0×10^{-3}	130
JM107 (pBAB56a, pBAB70)	1.0	9.0×10^{-3}	110
JM107 (pBAB56a, pBAB71)	1.0	7.0×10^{-3}	140
JM107 (pBAB56a, pBAB74)	1.0	8.0×10^{-2}	13
JM107 (pBAB56a, pBAB75)	1.1	7.0×10^{-2}	15

a Efficiency of plating is defined as: titer of the phage on strain x / titer of the phage on permissive host DH5αMCR (pBAB99).

b Restriction is defined as: restriction of non-methylated DNA / restriction of methylated DNA.

with McrC. JM107 (pBAB56a) transformed with either pBAB70 or pBAB71 exhibited restriction rescue with restriction 110 and 140 fold above the negative control, respectively. The restriction rescue effect observed with $McrB_S$ was still evident with truncated $McrB_S$ proteins lacking 28 amino acids, as in $McrB_S271$, and 118 amino acids, as in $McrB_S181$. This suggested that the region of $McrB_S$ that interacts with McrC was located, at least in part, within the N-terminal half of $McrB_S$.

Plasmids containing more extensive truncations of *mcrB*$_S$, pBAB74 or pBAB75 were unable to rescue restriction loss in JM107 (pBAB56a) and restriction was measured as 13 fold and 15 fold, respectively (Table 7). One interpretation of these data is that the N-terminal half of $McrB_S$ is required for interactions with McrC as defined by the restriction rescue assay. The C-terminal 118 amino acids do not appear necessary for interactions with McrC based on the truncated $McrB_S$ expressed by pBAB71.

(3) The McrB* restriction assay was used to explore the interaction between $McrB_L$ and $McrB_S$. In addition, the assay was used to examine the phenomenon of hyper-restriction observed with certain truncated forms of $McrB_S$. As stated earlier, overproduction of $McrB_S$ in DH5αMCR (pBAB43) caused decreased McrB* restriction and suggested $McrB_L$-$McrB_S$ interaction.

Starting with DH5αMCR (pBAB43) to express the $McrB_L$ protein, this strain was transformed with either pBAB70 to provide $McrB_S271$ or pBAB71 to provide $McrB_S181$. In the McrB* assay, the levels of restriction were equivalent to those observed with DH5αMCR (pBAB43, pBAB99) which provides $McrB_L$ and $McrB_S$

in equivalent amounts (i.e. no excess $McrB_S$ is present). This suggested that $McrB_S271$ and $McrB_S181$ had lost the ability to inhibit $McrB_L$ in the McrB* assay. It was believed that $McrB_S271$ and $McrB_S181$ could interact with $McrB_L$, but had lost their ability to "down regulate" based on results obtained with plasmids containing more extensive deletions of *mcrB*$_S$. DH5αMCR (pBAB43, pBAB74), which expressed $McrB_S65$, and DH5αMCR (pBAB43, pBAB75), which expressed $McrB_S35$ exhibited restriction 100 fold and 320 fold above the negative control, respectively. Presence of this hyper-restriction indicated that the peptides could still interact with $McrB_L$ and enhance restriction possibly by out competing endogenous $McrB_S$.

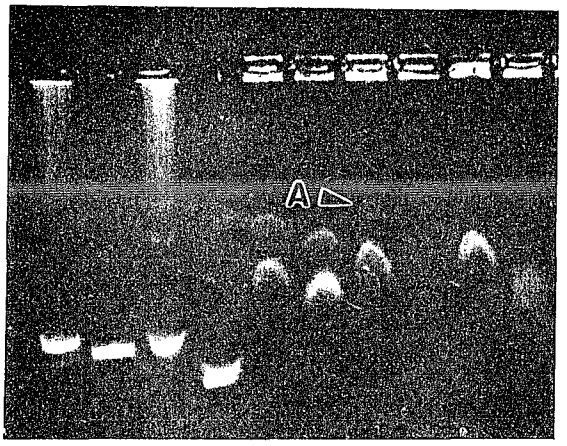
It should be noted that DH5αMCR (pBAB43, pBAB75) displayed an increased level of restriction of the non-methylated λ.O (Table 3). Although these are not high levels of restriction, they were repeatable and represented either a loss of specificity or magnification of a normally low level ability to cleave DNA lacking the appropriate methylation pattern. When the truncated $McrB_S$ series was tested with λ.*Bsu*RI in DH5αMCR (pBAB43), none of the strains exhibited altered restriction (Table 3). The phage λ.*Bsu*RI is not the substrate for McrB* activity of DH5αMCR (pBAB43). This suggests that hyper-restriction is a sequence dependent enhancement of the McrBC restriction system.

DH5αMCR (pBAB43,pBAB75) displayed an EOP of 0.44 with λ.*Bsu*RI. This phage possesses a methylation pattern that should not be restricted by a host possessing *mcrB* without *mcrC*, yet this seemed to exhibit some restriction. Upon further examination, it was noticed that λ.O is also slightly restricted by this host (Table 3). This suggests that this weak restriction is independent of the traditional

McrB* restriction recognition sites. It should be noted that DH5αMCR (pBAB43, pBAB74) displayed a slower than normal growth rate and, when viewed under phase-contrast microscope, a higher than normal amount of filamentous, non-septated cells. This was interpreted as evidence of DNA damage and the SOS response. DH5αMCR (pBAB43, pBAB75) was seen to possess exceptionally slow growth. After 3 days of growth in a 37°C shaker-incubator, these cells were viewed using a phase-contrast microscope. While cell shapes were pleomorphic, there was a conspicuous absence of filamentous cells. This may simply have been due to the exceptionally slow growth of these cells. Pleomorphic cell morphology, slow growth, and high levels of restriction of even non-methylated (i.e., λ.0) DNA are consistent with excessively high or nonspecific McrBC restriction activity.

Restriction results obtained using methylated λ were verified routinely using the methylase plasmid assay method (data not shown). This method consistently verified results (e.g., the documentation of hyper-restriction), but due to its extreme sensitivity, it was often difficult to obtain countable plates.

McrBC restriction *in vitro*. *In vitro* restriction of DNA using crude cell-free extracts was performed to verify that McrBC$^+$ strains were digesting DNA and that this restriction was dependent on methylation of specific sites. The necessity of GTP and subunit requirements was also examined. Agarose gel analysis showed that the methylated plasmid pM.*Bsu*RI20 was degraded by extracts from McrBC$^+$ strains, while the non-methylated plasmid pNM.*Bsu*RI20 was not (Fig. 9). This analysis demonstrated that GTP or ATP was necessary for digestion (Fig. 9). Without either,



Lane	1	2	3	4	5	6	7	8	9	10
DNA	NM	M	NM	M	NM	M	NM	M	NM	M
ATP or GTP	--	--	--	--	--	--	GTP	GTP	ATP	ATP

Fig. 9 - Digestion of pM.*Bsu*RI20 and pNM.*Bsu*RI20 using crude extracts. In the row labeled "DNA", NM designates pNM.*Bsu*RI20 and M designates pM.*Bsu*RI20. Lanes 1 - 6 contain no ATP or GTP. ATP or GTP was added to 0.3 mM where indicated. Lanes 1 and 2 contain linearized plasmid while lanes 3 and 4 contain uncut plasmid. Lanes 5 - 8 contain approximately 50 μg McrBC$^+$ crude extract. Note digestion of the band A in Lanes 8 and 10.

no digestion occurs. When GTP or ATP are added at 0.3 mM concentration, they seem to allow digestion equally well (Fig. 9, Lanes 8 and 10).

Since digestions used crude cell extracts, once cleaved, the DNA ends were exposed to non-specific exonuclease activity for the reaction time. If no DNA was seen in a given lane, it was assumed that McrBC had quickly cleaved all possible sites on the DNA and allowed degradation by exonucleases into small molecules of random length.

The GTP concentration of 0.6 mM allowed more digestion than did 0.3 mM. This finding was used to test the stability of McrBC under-reaction conditions. At the start of digestion 0.3 mM GTP was added and then an additional 0.3 mM GTP was added after 0.5 hours. This sample exhibited no digestion than a sample where only 0.3 mM GTP was added and the digestion allowed to proceed for one hour. This result suggested that McrBC is short lived or labile since no further digestion occurred in this reaction after 0.5 hours even though additional GTP was provided (results not shown).

Crude extracts made from a *mcrBC*$^+$ host also digested T4*gt* DNA, which possesses hydroxymethylcytosine. This was also readily accomplished using extract complementation where *mcrB*$^+$ extracts were insufficient to digest T4*gt* while they could be complemented by *mcrC*$^+$ extracts to exhibit significant digestion (Table 8). Extract complementation was not seen to exhibit significant digestion of pM.*Bsu*RI20. These results suggest that restriction of DNA containing 5-hydroxymethylcytosine (T4*gt*) is significantly more efficient than restriction of DNA containing 5-methylcytosine (pM.*Bsu*RI20). This conclusion agrees with results seen

Table 8. *In vitro* cleavage of pM.*Bsu*RI20 and T4 *gt* DNA using extracts made from mutants of the McrBC system

	DNA[a]	Extract[b]	Restriction[c]
1	NM + M	B^+	-
2	NM + M	B^+, C^+	-
3	T4	B^+, C^+	-
4	T4 *gt*	B^+	-
5	T4 *gt*	B^+, C^+	+
6	NM, M	p11	-
7	NM, M	p11, C^+	-
8	T4	p11, C^+	-
9	T4 *gt*	p11	-
10	T4 *gt*	p11, C^+	+
11	NM, M	p12	-
12	NM, M	p12, C^+	+
13	T4	p12, C^+	-
14	T4 *gt*	p12	+
15	T4 *gt*	p12, C^+	+

a This lane represents DNA used in this assay. NM represents pNM.*Bsu*RI20; M represents pM.*Bsu*RI20, and in each case pNM.*Bsu*RI20 added as a control was not digested; T4 represents wild-type T4 DNA; and T4 *gt* represents non-glycosylated T4 DNA.

b Approximately 40 μg of each cell free extract listed was used. Represented are: B^+ which signifies extract derived from a $McrB^+$ host; C^+ which represents extract derived from a $McrC^+$ host; p11 which represents extract prepared from hosts containing pRAB11, which produces truncated $McrB_L$ and $McrB_S$; p12 which represents extract made from a host containing pRAB12, which produces $McrB_L$, $McrB_S$, and truncated McrC.

c The minus (-) symbol represents no cleavage, and the plus (+) symbol represents cleavage of pM.*Bsu*RI20 or phage T4 *gt* DNA.

with the *in vivo* McrBC and Rgl restriction assays. This may be due to the range of different modified sequences present in T4*gt* DNA.

The plasmid pRAB11 (68) produces truncated $McrB_L$ and $McrB_S$. Extracts produced from a host possessing pRAB11 were not able to digest T4*gt* DNA. But when extract prepared using a pRAB16 (McrC)-possessing host was added to the pRAB11 extract, significant digestion occurred (Table 8). This suggests that truncated $McrB_L$ and $McrB_S$ can be complemented by McrC to yield restriction of DNA possessing hydroxymethylcytosine. This agrees with the "restriction rescue" assay in which pBAB71, a plasmid that produces the same truncated $McrB_S$ as pRAB11, was able to bind to McrC.

Extract produced from a host possessing pRAB12 (65) was used for *in vitro* digestions also. Plasmid pRAB12 produces both *mcrB* gene products and a truncated McrC peptide lacking one-third of the C-terminal end. Cleavage of neither pNM.*Bsu*RI20 or the methylated pM.*Bsu*RI20 occurred using this extract alone. However, extract containing authentic McrC did complement the extracts made from a pRAB12 containing host. The plasmid pM.*Bsu*RI20 exhibited cleavage, while pNM.*Bsu*RI20 did not. These results indicate that the non-methylated plasmid was not cleaved by any extract combinations, while the methylated plasmid was cleaved when authentic $McrB_L$, $McrB_S$, and McrC were produced by certain vectors. This suggests that truncated McrC is insufficient to function as the specificity subunit when the target DNA contains ^{m5}C. Extract from pRAB12 containing host was sufficient to cause cleavage of T4*gt* suggesting even though McrC has been truncated

it can still function as a specificity subunit well enough to identify T4*gt* DNA as a McrBC target.

In vitro restriction digestions were performed using plasmids containing one or two sites methylated by M.*Pvu*II as digestion substrates (Fig. 10). The plasmids pBAB99, pBR322, and pBR322 with an *Eco*0109I deletion each contain one *Pvu*II site, while pUC18 contains two *Pvu*II sites separated by 326-bp. All plasmid substrates were seen to undergo cleavage (Fig. 10, Lanes 2, 4, 6, and 8). In carefully controlled experiments, the linear DNA band generated from a once cut plasmid is clearly visible. Not all methylated *Pvu*II sites cleaved equally. The methylase plasmid pM.*Pvu*II was present in each plasmid preparation seen in Fig. 10 and contains two *Pvu*II sites. Limited digestion of pM.*Pvu*II was observed relative to the target plasmids (Fig. 10). In a competition digestion study, two methylated target plasmids were digested in the same reaction. The relative digestion of each target was quantified and compared to all combinations of target plasmids. In terms of efficiency of digestion the relative order was: pBR322=pBR322 with an *Eco*0109I deletion > pBAB99 > pUC18 > pM.*Pvu*II20 sites. This site preference was also observed in Fig. 10. In Fig. 10, when the pBR322 derived methylated *Pvu*II sites were present, these sites out-competed pM.*Pvu*II20 sites for the McrBC endonuclease. Linearized pM.*Pvu*II20 bands were present in reactions with the methylated *Pvu*II sites of pUC18 and pBAB99. The site from pBR322 and the hybrid *Pvu*II site created in pBAB99 were chosen as targets for additional studies. The successful cleavage of these DNA substrates led to their

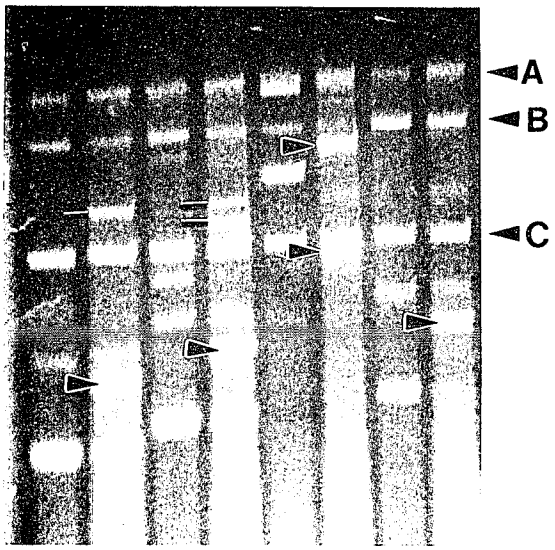


Fig. 10 - *In vitro* digestion of plasmids containing methylated *Pvu*II sites. Samples in Lanes 1, 3, 5, and 7 were treated with extract from McrBC$^-$ cells, while samples in Lanes 2, 4, 5, and 8 were treated with extract from McrBC$^+$ cells. Lanes 1 and 2 contain pBAB99. Lanes 3 and 4 contain pUC18. Lanes 5 and 6 contain pBR322, and Lanes 7 and 8 contain pBR322 with the *Eco*0109I deletion. The arrows mark the location of linear forms of the plasmids. In Lane 6, the two arrows mark linear forms of the dimer and monomer. Each lane also contains the plasmid pM.*Pvu*II20 which was used to methylate the plasmids *in vivo*. The arrow marked "A" is chromosomal DNA; "B" is the pM.*Pvu*II20 dimer; and "C" is the pM.*Pvu*II20 monomer. The dashes (-) are linearized pM.*Pvu*II20.

use in the construction of target substrates used in the McrBC-DNA UV cross linking assay.

Gel retardation and UV-cross linking assays. To examine the interaction between the McrBC restriction enzyme complex and its DNA substrate, gel retardation was chosen to separate unbound target DNA from DNA complexed to the protein complex. Since the enzyme preparation was to be crude protein preparations from *E. coli* strains, it was essential to use defined target DNA fragments. Based on initial experiments with *in vitro* DNA cleavage with McrBC extracts, two *Pvu*II sites were chosen that could be specifically methylated by M.*Pvu*II encoded by the plasmid pM.*Pvu*II20. One site was the *Pvu*II site from pBR322 and the other was the hybrid site created upon deletion of the α-complementation region of pUC18 using flanking *Pvu*II sites (i.e. the single *Pvu*II site found in pBAB99). In addition to testing each site as a single site, the hybrid site present in pBAB99 was subcloned as a 37-bp DNA fragment with three copies in tandem and with two copies in inverted orientation (Figs. 11, 12). Nucleotide sequencing confirmed that pBAB63.1 contained three copies of the 37-bp fragment in tandem with the three *Pvu*II sites 37-bp apart. Plasmid pBAB63.11 had two copies of the 37-bp fragment in inverted orientation and the two *Pvu*II sites were 34-bp apart.

Using a standard gel retardation protocol, cells extracts from the restrictionless strain ER1648 (pBAB99), *mcrBC, mcrA, mrr, hsdRMS,* and the McrBC restriction strain ER1564 (pBAB9), *mcrA, mrr, hsdRMS* were tested for the ability to bind the three-site target end-labeled with ^{32}P. To prepare the cell extracts,

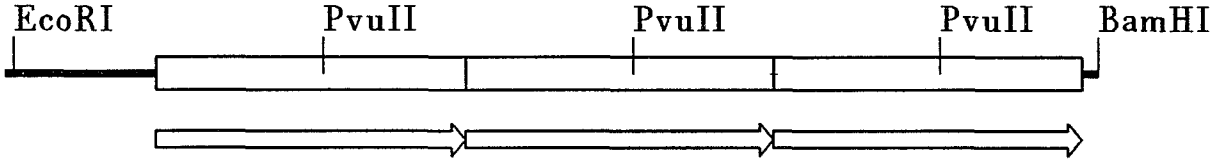


Fig. 11 - The 131-bp target fragment of pBAB63.1. Each of the three 37-bp cassettes that make up the 131-bp *Eco*RI-*Bam*HI fragment are in the same orientation. There are 37-bp between each of the three sequential *Pvu*II sites. The sequence of the 37-bp cassette is as follows: 5'-CCTCTTCGCTATTACGCCAGCTGCATTAATGAATCGG-3'. The *Pvu*II site is underlined.

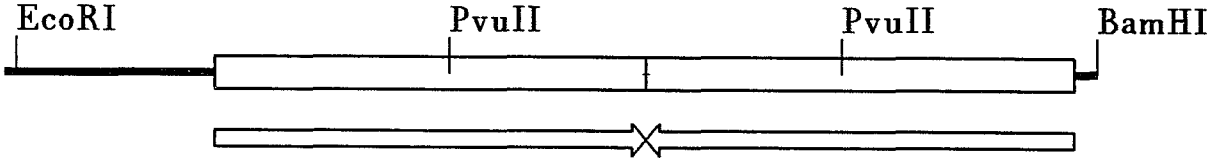
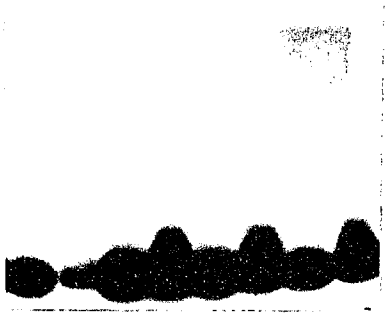


Fig. 12 - The 94-bp target fragment of pBAB63.11. The two 37-bp cassettes that make up the 94-bp *Eco*RI-*Bam*HI fragment of pBAB63.11 are in opposite orientations. This positions the two *Pvu*II sites 34-bp apart. The sequence of the cassette is the same as in Fig. 11.

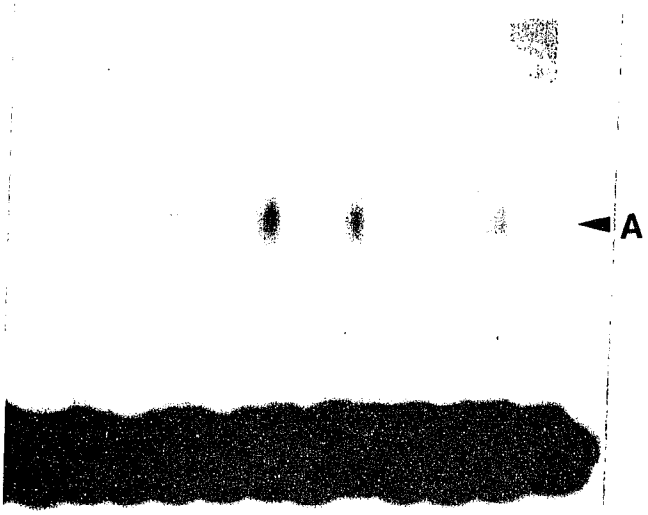
cultures were inoculated to an OD_{600} of approximately 0.2 and grown to stationary phase overnight. To prevent McrBC digestion and block other nucleases in the extract, EDTA was added to chelate endogenous magnesium ions during the binding reaction. No gel shift was observed using cells in early, mid, or late log phase growth. The initial gel shift observed was so small that it did not appear to represent the bound complex (Fig. 13). However, the barely perceptible shifted DNA "band" observed in Lanes 5, 7, and 9 could be competed away with an excess of unlabeled methylated target DNA. In addition, the extent of band shift observed after 15 min. of electrophoresis was indistinguishable from that present after 2.5 hours of electrophoresis (data not shown). This was taken as evidence that the McrBC complex was extremely labile and quickly denatured upon entering the gel matrix or that the gel composition prevented reassociation after initial binding and release. Based on this hypothesis, McrBC complex did bind specifically to the DNA target and should then be susceptible to cross linking to the target.

UV light at 254nm was used to cross link proteins to DNA in the binding reaction. By examining the cross linked complex at various times of exposure to UV light, times of 10 or 15 min. were sufficient to accumulate detectable complex (Fig. 14, Lanes 7, 9, 11, and 14). The complexes were shown to be specific to McrBC-containing extracts, resistant to non-methylated competitor target DNA and highly susceptible to the methylated target used as an unlabeled competitor. It should be noted that competition with unlabeled DNA occurs in the binding reaction prior to UV cross linking.



Lane	1	2	3	4	5	6	7	8
Extract	N/A	N/A	-	+	-	+	-	+

Fig. 13 - Gel shift assay. The end labeled 131-bp target fragment of pBAB63.1 was used in this assay. Lanes 3, 5, and 7 contain 12, 18, and 24 μg of extract from McrBC$^-$ cells, respectively. Lanes 4, 6, and 8 contain 10, 15, and 20 μg of extract from McrBC$^+$ cells, respectively. Lane 1 had no extract added, while Lane 2 was empty. The minus (-) and plus (+) signs refer to binding reactions using extracts from McrBC$^-$ and McrBC$^+$ hosts, respectively.



Lane	1	2	3	4	5	6	7	8	9	10	11	12
Extract	-	+	-	+	-	+	-	+	+	+	+	+
Min.	0	0	5	5	10	10	15	15	5	5	10	10

Fig. 14 - McrBC-DNA crosslinking with UV light. The end-labeled 131-bp target fragment of pBAB63.1 was used in this assay. In the row marked "extract" each (-) represents 15 μg extract from a McrBC$^-$ host, while each (+) represents 15 μg extract from a McrBC$^+$ host. The row marked "Min." represents time in minutes for UV irradiation. Lanes 9 and 11 had 1.5 μg non-specific competitor DNA added (pUC19), while Lanes 10 and 12 had 1.5 μg methylated specific competitor added (pBAB63.1). Note the specific McrBC-DNA complex band designated "A".

The conditions that would permit efficient binding were defined. Weak protein-DNA interactions are greatly affected by the ionic conditions of *in vitro* reactions. Maximum, specific binding to the target DNA was observed at approximately 50 mM KC1 concentration (Fig. 15). The requirement of GTP for McrBC activity has been proposed (76). To see if GTP is necessary for protein binding or only for DNA cleavage, binding reactions were set up with GTP added to final concentrations of 1.0, 0.5, 0.1, 0.05, and 0.0 mM. As GTP decreased, the specific shift cross linked complex became more diffuse and appeared to separate into multiple bands (Fig. 16). It should be pointed out that internal cell GTP concentrations are approximately 1 mM (13, 76) and, although it is stated that no GTP was added to one sample, there was still some amount of diluted cellular GTP. To confirm this, when 10 mM $MgCl_2$ was added in the absence of added GTP as in Fig. 16, Lane 11, the McrBC-cross linked target fragment band was completely lost, while non-specifically cross linked bands were not.

Using the M.*Pvu*II methylated targets, expression of the entire *mcrBC* operon was necessary to see the cross linked complex with cell extracts. The complex was not seen from extracts possessing $McrB_S$ only, $McrB_L$ and $McrB_S$, $McrB_S$ and McrC, the truncated *mcrB* gene products expressed by pRAB11 (68) or from McrC alone. A weak cross linked complex band was observed with expression of *mcrB* alone, however, these complexes were not tight enough to resist competition with non-methylated DNA. This may suggest that $McrB_L$ can bind N4-methylcytosine-containing DNA but not strongly. This agrees with *in vivo* restriction and *in vitro* restriction assays which indicate that the entire McrBC complex is needed for

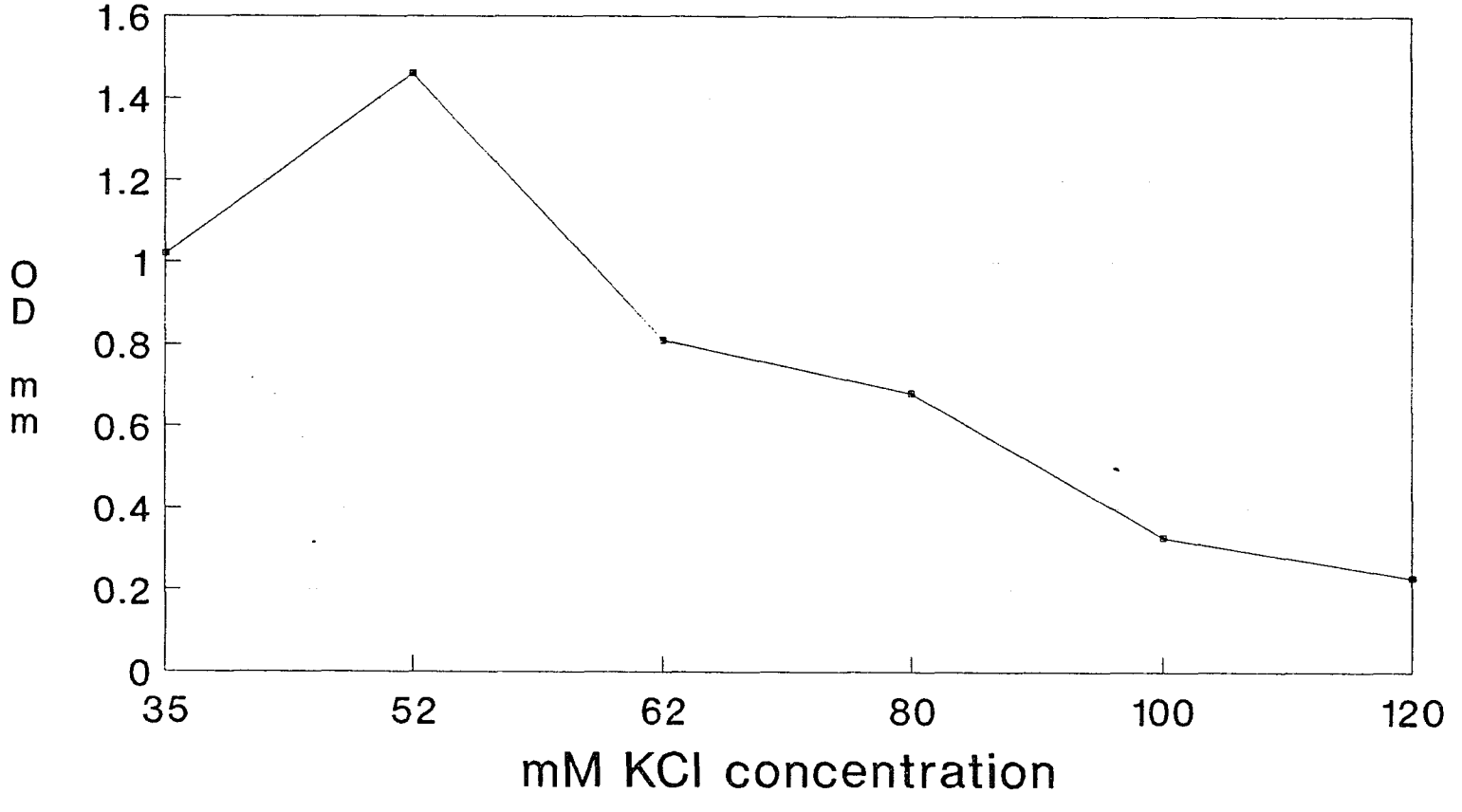
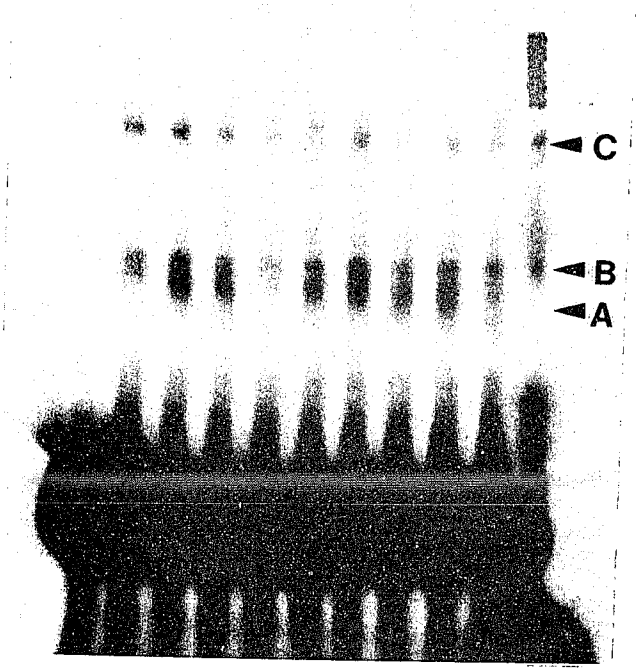


Fig. 15 - Effect of KCl concentration on formation of McrBC-DNA complex bands from the UV cross linking assay. Density of exposed silver grains on autoradiograms were quantified in the same axis as electrophoresis. Cumulative density for the McrBC-DNA complex is represented in OD_{mm}.



Lane	1	2	3	4	5	6	7	8	9	10	11
Extract	N/A	-	+	+	+	+	+	+	+	+	+
mM GTP Conc.	1.0	1.0	1.0	1.0	1.0	1.0	0.5	0.1	.05	0.0	0.0

Fig. 16 - Protein-DNA UV cross linked bands obtained as a function of GTP concentration. The end labeled 131-bp target fragment of pBAB63.1 was used in this assay. In the row marked as Extract, 15 μg extract from a McrBC$^-$ host is designated as (-) and 15 μg extract from a McrBC$^+$ host is designated as (+). Lane 4 contains 0.5 μg non-specific competitor (pUC19), and Lane 5 contains 0.5 μg methylated pBAB63.1. The region of the McrBC-dependent band is marked by "A". Bands "B" and "C" were formed with all extracts. Lane 11 received 10 mM $MgCl_2$ to promote cleavage of DNA in specific complexes.

restriction of N4-methylcytosine-containing DNA. For this reason, further analysis of DNA binding was done with strains expressing the complete *mcrBC* operon.

Distinct bands were obtained with the methylated radiolabeled target fragments from pBAB63.1 (i.e., containing the three *Pvu*II sites) and pBAB63.11 (i.e., containing the two-site target fragment) (Fig. 17). When ER1648 (pBAB99) extract was used in the cross linking assay no specific complex band was observed. ER1564 (pBAB9) extract exhibited an McrBC-dependent band present upon UV cross linking. This specific band was not competed by an excess of unlabeled, non-methylated target DNA and was eliminated upon competition with an excess of unlabeled, methylated target DNA (Fig. 17). This series confirmed that the band represented a specific complex between McrBC and the methylated target DNA. Proximal *Pvu*II sites in the target fragment of pBAB63.1 are 37-bp apart, while distal *Pvu*II sites are 74-bp apart. The *Pvu*II sites in pBAB63.11 are 34-bp apart. This indicated that McrBC was either bound to individual methylated sites or bound to multiple sites with diverse spacing. Since McrBC-dependent linearization of plasmids containing single methylated *Pvu*II sites was observed, single-site target fragments (Fig. 18) were tested in the cross linking assay. Interestingly, McrBC-dependent cross linking to single-site target DNA fragments was more intense that the two- or three-site targets (Fig. 17). However, neither single-site target fragment from pBAB61 or pBAB62 gave a defined band. The smeared band was McrBC dependent, resistant to non-methylated competitor DNA, and highly sensitive to methylated target DNA competitor. It is thought that the active restriction complex possesses two DNA binding sites. The smeared bands may represent a combination

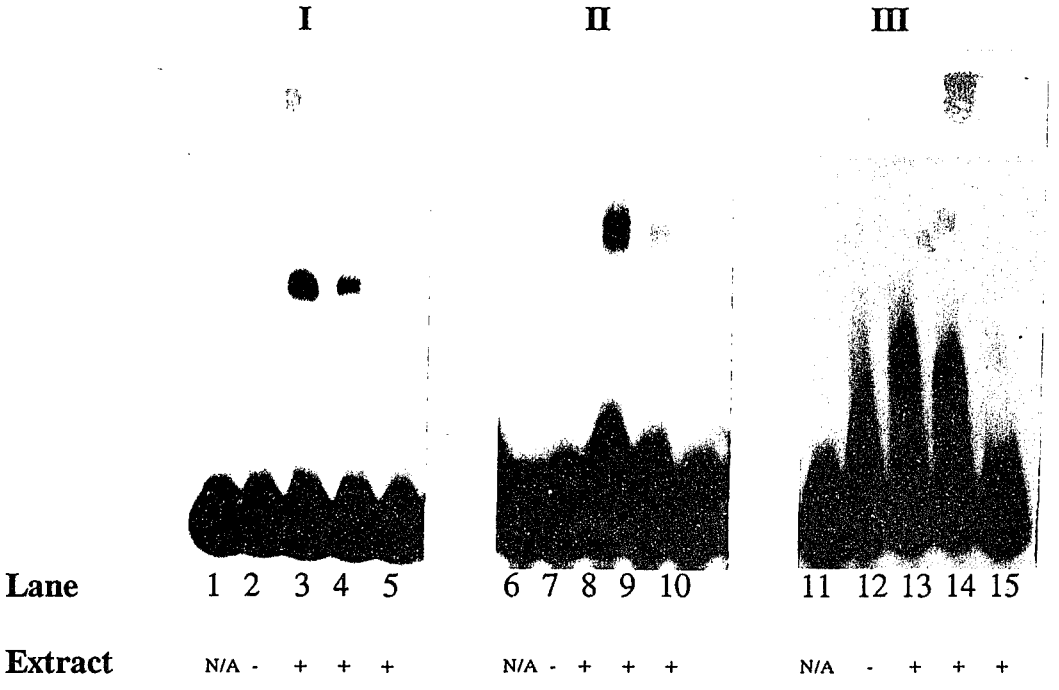


Fig. 17 - McrBC-DNA UV cross linking of target fragments from pBAB63.1, pBAB63.11, and pBAB61. *Group I consists of the 131-bp target fragment of* pBAB63.1. Group II consists of the 94-bp target fragment of pBAB63.11, and group III consists of the 170-bp target fragment of pBAB61. In the row marked Extract, N/A signifies no extract added. The (-) signifies 15 μg extract from a McrBC$^-$ host. The (+) signifies 15 μg extract from a McrBC$^+$ host. Lanes 4, 9, and 14 each contain 0.5 μg non-specific competitor DNA (pUC19), and Lanes 5, 10, and 15 each contain 0.5 μg methylated specific competitor DNA, pBAB63.1, pBAB63.11, and pBAB61 for groups I, II, and III, respectively.

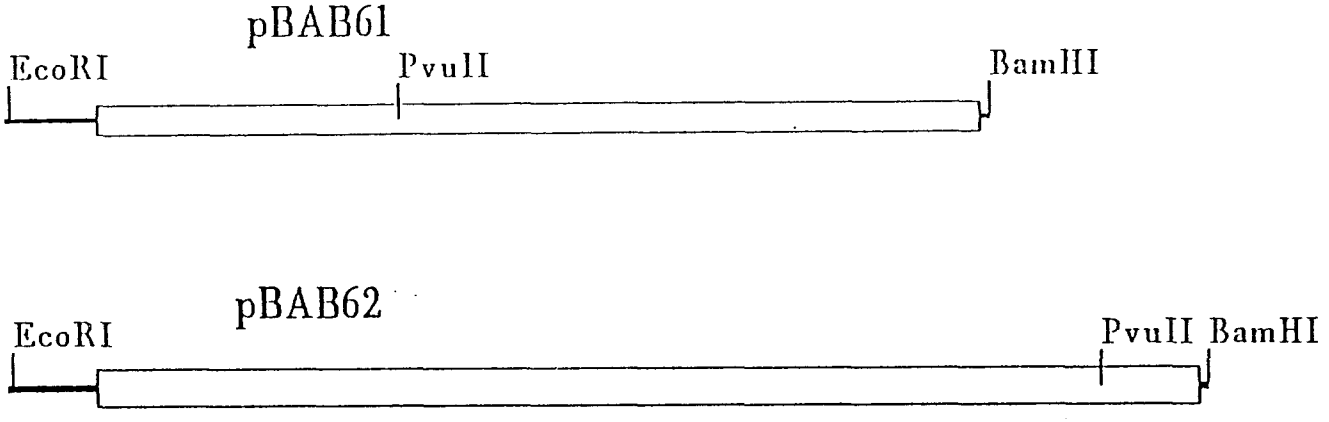


Fig. 18 - The single *Pvu*II site-containing fragments of pBAB61 and pBAB62. The 191-bp *Eco*RI-*Bam*HI fragment of pBAB61 possesses one *Pvu*II site situated 70-bp from one end and was derived from pBR322. The 243-bp *Eco*RI-*Bam*HI fragment of pBAB62 contains a single *Pvu*II site situated 22-bp from the fragment end. The *Pvu*II site of pBAB62 was the hybrid site created in pBAB99.

of (i) McrBC molecules cross linked to one or two target DNA molecules, and (ii) non-cross linked, gel shift complexes due to protein binding. Together these complexes may be visualized as a smear. OD_{nm} readings obtained from Fig. 17 using densitometry for lanes 12-15 are as follows: Lane 12 - 0.56, Lane 13 - 2.18, Lane 14 - 2.13, and Lane 15 - 0.19. These results clearly suggest McrBC-DNA complex are present in lanes 13 and 14.

To confirm that DNA containing a single methylated site could bind McrBC, single-site targets were used as unlabeled competitor for the labeled two- and three-site targets. It was observed that excess methylated pBAB61 (i.e., containing a single copy of the hybrid *Pvu*II site found in pBAB99) could be used as specific competitor to eliminate a specific McrBC cross linked band, while the same target fragment, when non-methylated, did not serve as competitor (results not shown). This result confirmed that a single site methylated site could bind McrBC and that binding was methylation dependent.

DISCUSSION

McrBC subunit function. The 53-kDa $McrB_L$ peptide functions as the restriction endonuclease of the McrBC restriction system (23, 24, 36). The 38-kDa McrC peptide is necessary for efficient cleavage of most methylated targets (23, 24, 66). Data supporting these findings were generated using antisense RNA designed to lower translation of each peptide. ASR1 caused underexpression of $McrB_L$ and lowered McrBC and Rgl restriction. This was entirely expected as less endonuclease should result in less restriction. ASR3 caused underexpression of McrC and concomitantly McrB and Rgl restriction were lowered. Lowered levels of a subunit necessary for target recognition was expected to lower McrBC and Rgl restriction. ASR3, however, was not expected to lower the McrC-independent McrB* restriction. This was shown to be true as JM107 (pASR3), when compared with JM107 (pARV11), exhibited a decrease in restriction of λ.*Bsu*RI but no loss in restriction of λ.SPR. In addition, *mcrBC*$^+$ cells possessing ASR1 or ASR3 exhibited no secondary effects and appeared normal except for lowered restriction levels. All of these facts support existing hypotheses with regards to $McrB_L$ and McrC.

Function of $McrB_S$ in the McrBC restriction system. The role of $McrB_S$ was unknown. It has no endonuclease activity alone or with McrC. However, it is not possible to mutate $McrB_S$ without affecting $McrB_L$. In this study, we were able to alter the levels of $McrB_S$ without altering $McrB_L$.

The two McrB proteins have been shown experimentally to be expressed in approximately equimolar amounts (24, 65, 66). It has also been suggested that McrC is expressed at one-third the molar amount of $McrB_L$ or $McrB_S$. This 3:3:1

ratio ($McrB_L$:$McrB_S$:McrC) may serve to set appropriate restriction levels *in vivo*. Our data suggested that the relative amount of $McrB_S$ to $McrB_L$ or McrC is especially relevant. When the level of $McrB_S$ relative to $McrB_L$ or $McrB_S$ was dramatically altered, restriction levels were altered. When $McrB_S$ was overproduced, McrBC and Rgl restriction were diminished or eliminated. The loss of restriction correlated with an excess $McrB_S$. Based on Western blots, when $McrB_S$ was overproduced, the $McrB_L$ levels remained constant. Since restriction decreased while $McrB_L$ levels remained constant, $McrB_S$ appeared to inhibit restriction at the level of protein-protein interactions. In addition, when $McrB_S$ was underproduced using ASR2, McrBC and Rgl restriction were disrupted and the SOS response was triggered. These findings support the idea that $McrB_S$ can interact with and inhibit the McrBC complex.

In a host possessing *mcrB* alone, overproduction of $McrB_S$ was also shown to diminish McrB* restriction. This suggested that $McrB_S$ could interact with $McrB_L$ since there was no McrC present. This further suggested that $McrB_S$ was a regulatory protein that disrupted active complex formation. With the thought that $McrB_S$ could regulate restriction, a series of DNA constructs was made possessing sequentially greater truncations of *$McrB_S$*. The purported $McrB_S$ truncations were used to study the ability of $McrB_S$ to interact with $McrB_L$ as measured by McrB* restriction. When overproduced in wild-type host (JM107), authentic $McrB_S$ caused restriction to decrease. The smaller truncations of $McrB_S$ caused this peptide to lose its ability to inhibit McrBC and McrB* restriction. When assayed for restriction, the same host possessing greater truncations of $McrB_S$ displayed hyper-restriction.

Hyper-restriction occurred when the smallest of the $McrB_S$-truncated peptides were overexpressed and displaced the endogenous $McrB_S$ peptide. In the presence of these short peptides, the ability of $McrB_S$ to modulate formation of active restriction complexes was negated and more of the active complexes were formed. This phenomenon will be revisited, below, in terms of model development.

$McrB_S$ is produced by *mcrB* from an inframe, internal translational start. This results in $McrB_S$ possessing an amino acid sequence identical to the carboxyl-terminal 65% of $McrB_L$. There is precedence for regulator proteins produced from inframe, internal translation starts. The bacteriophage f1 possesses a regulator protein produced in this manner (29). The gene II protein product is necessary for all f1 DNA synthesis other than the initial synthesis of the complement of the incoming (+) strand of phage DNA. The gene X protein (C-terminal fragment of gene II, started at an inframe AUG within gene II) inhibits gene II protein activity. When overexpressed in *trans*, the gene X protein blocks all phage-specific DNA synthesis.

Another example of this phenomenon occurs with the transposon Tn5. It is known that transposition of Tn5 is regulated by a peptide produced from an inframe, internal translational start of the IS50R mRNA. The full length peptide is a transposase, while the 40 amino acids shorter peptide functions as a transposase inhibitor (33). By producing the shorter, inhibitory peptide in four times the amount of the transposase, transposition is effectively reduced to levels tolerated by the cell. Tn5 normally transposes upon introduction to a new host since the shorter, inhibitory peptide has not accumulated in the cell. It was shown that the coding region of the

shorter peptide was sufficient, when produced in *trans*, to block the transposition of newly introduced Tn5. In an analogous fashion, $McrB_S$, when overproduced in *trans*, blocked McrBC activity. And just as unregulated transposition of Tn5 would kill the *E. coli* host, evidence from the present study indicated that unregulated McrBC activity damages DNA and induces the SOS response and often cell death.

The favored model for the inhibition of Tn5 transposase by the shorter peptide is based on protein-protein interactions and the formation of inactive hetero-oligomers with the transposase and the shorter peptide. This type of model offers the best agreement with the results of the present study of McrBC restriction.

Model describing the interaction of subunits in the McrBC restriction system. Interpretation of our data led us to believe that $McrB_S$ was a regulator of the McrBC restriction system. These findings were also used to construct a model to describe the active McrBC restriction complex. The first finding that contributes to model development is that $McrB_S$ interacts with $McrB_L$, the endonuclease. Based on results of McrB* assays, overproduction of $McrB_S$ or truncated versions of $McrB_S$ alter the ability of $McrB_L$ to restrict the methylated target DNA in the absence of McrC. It is assumed that if $McrB_S$ and $McrB_L$ interact, $McrB_L$, which contains all the amino acids of $McrB_S$, can interact with itself. For this reason we propose that $McrB_L$ can form minimally a dimer. Since $McrB_S$ can disrupt the function of $McrB_L$ in the McrB* assay, it is proposed that the $McrB_S$-$McrB_L$ heterodimer is inactive and that the $McrB_L$ homodimer is the active endonuclease (Fig. 19).

Relative amounts of $McrB_L$ and $McrB_S$ in the cell are approximately equal. Using the assumption the $McrB_L$ and $McrB_S$ bind each other equally, simple

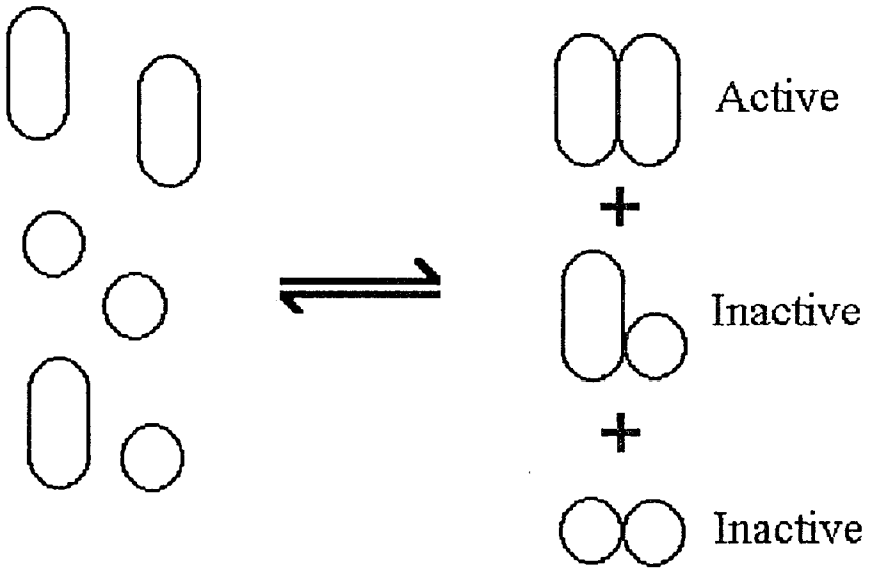


Fig. 19 - Random chance interaction between $McrB_L$ and $McrB_S$. $McrB_L$ is represented by an ovoid, while $McrB_S$ is depicted by the white circle. Chance interaction between the subunits displayed would result in the active $McrB_L$ homodimer forming 25% of the time. $McrB_L$-$McrB_S$ heterodimers and $McrB_S$ homodimers are proposed to have no restriction activity.

probability calculations can be used to theorize that 50% of the $McrB_L$ subunits will be in active $McrB_L$ homodimer while the other half will be tied up in non-functional heterodimers with $McrB_S$. The remaining $McrB_S$ would be found in homodimers with itself. Thus of the peptides produced from the *mcrB* gene, 25% would be tied up in active $McrB_L$-$McrB_L$ homodimers, 50% in inactive $McrB_L$-$McrB_S$ heterodimers, and 25% in inactive $McrB_S$ homodimers (Fig. 19). This pattern of subunit binding would explain how $McrB_S$ is able to modulate or regulate the levels of McrBC activity by limiting the amount of active complex formed. This $McrB_S$-dependent buffering of McrBC restriction is even more evident when McrC is incorporated into the model.

In vitro studies with purified $McrB_L$ and McrC (76) suggest that these two proteins are required to form an active restriction complex. Our studies suggested that McrC can interact with $McrB_S$ and $McrB_L$. When McrC was overexpressed in JM107, McrBC and Rgl restriction were diminished or eliminated. This indicated that McrC can interact with the $McrB_L$-containing active endonuclease complex and that too much McrC disrupts this complex. When $McrB_S$ and McrC were overexpressed together there was no loss of restriction (i.e., restriction rescue occurred). This evidence suggests that $McrB_S$ and McrC interact. Since $McrB_L$ contains all the amino acids found in $McrB_S$, it seems likely that these peptides share the McrC binding site. The model for subunit interaction predicts that McrC can bind each of the McrB complexes proposed in Fig. 19. $McrB_S$ would thus effectively lower the amount of McrC available to the active $McrB_L$ homodimers and further serves as a restriction "down regulator" *in vivo* (Fig. 20). It should be noted

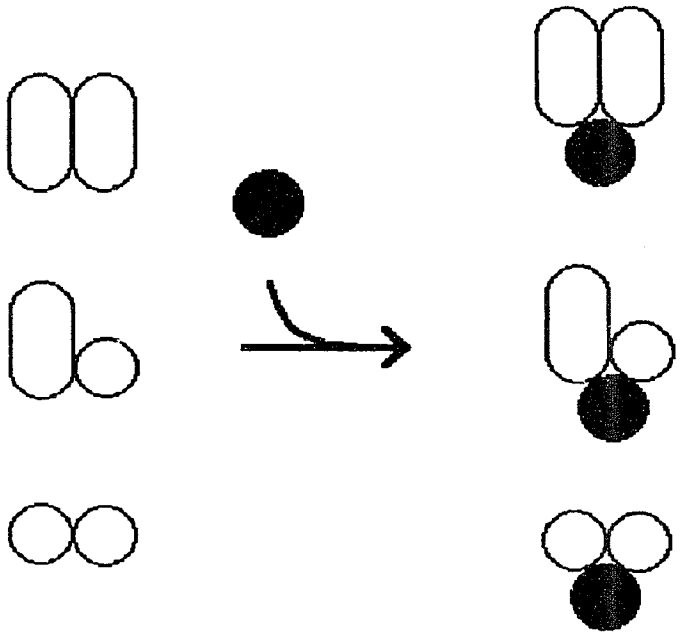


Fig. 20 - The interaction of McrC with the *mcrB* gene products. The ovoid figure represents the $McrB_L$ subunit. The open circle represents $McrB_S$ and the filled circle represents McrC. The proposed model specifies that a single McrC monomer interaets with the $McrB_L$ homodimer to yield the active McrBC complex. Other multimeric complexes binding McrC are inactive.

that given a $McrB_L$:$McrB_S$:McrC ratio of 3:3:1, only one of the $McrB_L$ homodimers in three would be predicted to have an McrC subunit bound. Based on the molar ratio of subunits, it was initially assumed that one McrC would bind an $McrB_L$ homodimer to make an active complex. This subunit arrangement of one McrC for two McrB subunits would also explain the inhibition of McrBC activity upon overexpression of *mcrC*.

Overexpression of the purported specificity subunit, McrC, lowers McrBC and Rgl restriction. The level of McrC needed to disrupt restriction was tested using various vectors. Inhibition was observed when *mcrC* was expressed from pUC8 at 200 copies/chromosome (pRAB17), pBR322 at 60 copies/chromosome (pBAB56a), or pECA310, a pPvuI derivative at 5 copies/chromosome (pBAB46a). This suggests that even low levels of McrC overexpression cause loss of restriction. The model explains inhibition of McrBC by excess McrC by assuming that each McrB subunit has an McrC binding site. The model would predict that the binding of two McrC subunits per McrB unit dimer disrupts the complex (Fig. 21). An increase in available McrC may allow this subunit either to bind $McrB_L$ monomers to create an inactive $McrB_L$-McrC complex or that two McrC proteins bind to and inactivate the $McrB_L$ homodimer.

It is thought that low-level expression of McrC in proper proportion to McrB subunits is necessary for maintenance of proper subunit ratios that will yield the active restriction complex. One strategy used in nature to insure that the proper ratio of proteins is made is translational coupling. Sequence analysis of the *mcrB* and *mcrC* genes suggested that McrC synthesis may be translationally coupled to

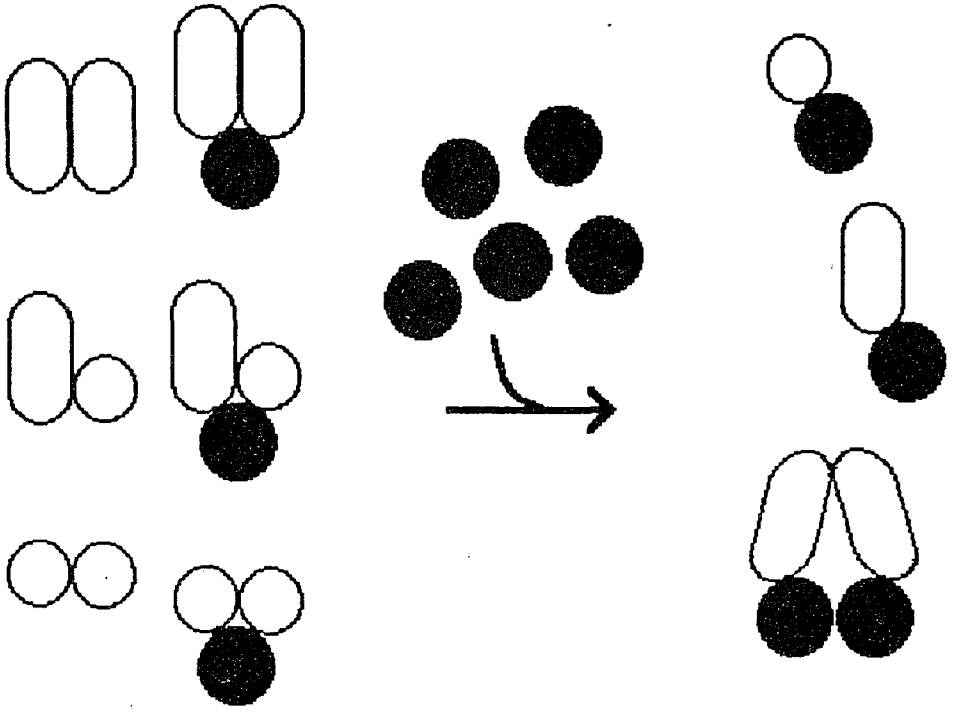


Fig. 21 - Overexpression of McrC. When McrC, which is represented by the filled circle, is overproduced, restriction loss occurs. According to the model, excess McrC binds $McrB_L$ monomers preventing dimerization or because two McrC proteins bind the $McrB_L$ homodimer creating an inactive complex. The ovoid represents $McrB_L$ and the open circle represents $McrB_S$.

McrB (66). In another study efficient overproduction of McrC for purification was only achieved when McrC synthesis was translationally coupled to a hybrid McrB protein (88). It appears likely that low-level McrC expression is controlled by *mcrB-mcrC* translational coupling. Given a $McrB_L$:$McrB_S$:McrC ratio of 3:3:1, it is thought that 6 peptides must terminate at the end of *mcrB* ORF for each peptide initiated at the *mcrC*-coding region (67).

A good example where translational coupling regulates expression of a second peptide to maintain low levels is the *ompB* loci. This region consists of the translationally coupled *ompR* and *envZ* genes. These genes function to regulate outer membrane proteins in response to changes in environmental osmolarity. The *envZ* gene produces membrane-bound sensor proteins and must be expressed at low levels. Too much expression from *envZ* makes the cell overly sensitive to environmental osmotic changes. Low level expression is insured by translational coupling (50).

Use of the model to explain inhibition of McrBC by overproduction of $McrB_S$. The overproduction of $McrB_S$ effectively eliminated McrBC restriction as assayed by the McrBC assay or the Rgl assay. The proposed model predicts that additional $McrB_S$ would greatly increase the amount of the inactive $McrB_L$-$McrB_S$ heterodimer at the expense of the active $McrB_L$ homodimer (Fig. 22). While it is true that overproduction of $McrB_S$ in JM107 yields loss of restriction, overproduction of $McrB_L$ and $McrB_S$ together yield no such loss. This is because more active and inactive complexes are formed in the normal proportion for a net result of slightly increased restriction. Since McrC is limiting a large increase in restriction activity would not be expected. As stated previously and in keeping with the proposed

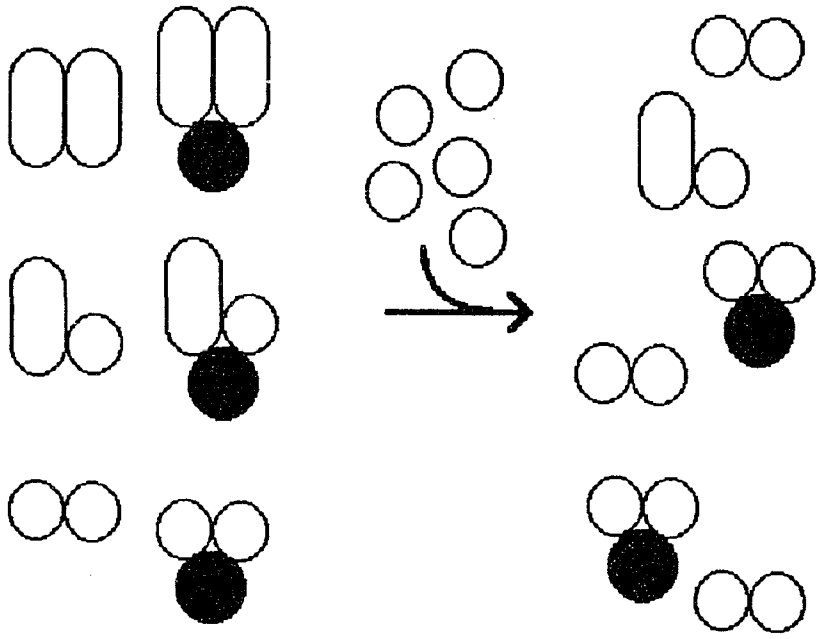


Fig. 22 - Overproduction of the $McrB_S$ peptide. The ovoid represents the $McrB_L$ subunit, while the open circle represents $McrB_S$. The filled circle represents McrC. Restriction loss occurs when $McrB_S$ is overproduced. The model states that this peptide in excess disrupts active $McrB_L$ homodimer formation and shifts the complex formation equilibrium towards a greater number of inactive $McrB_L$-$McrB_S$ heterodimers. $McrB_S$ is also proposed to dimerize and may bind McrC to form inactive complexes.

model, when $McrB_S$ is overproduced in a strain possessing only *mcrB*, McrB* restriction is diminished.

To this point, it has been assumed that $McrB_L$ and $McrB_S$ bind themselves and each other with the same affinity. If these two subunits bind each other equally, when pRAB17 was used to overproduce $McrB_S$ in the wild-type host JM107, there would be virtually no active complex formed. $McrB_S$ was thought to be 320-fold in excess with JM107 (pRAB17). This would allow a $McrB_L$ homodimer to form so seldom as to drop restriction below measurable levels. This is not the case as restriction 3 fold above the negative control was retained. This evidence suggested that the $McrB_L$ homodimer is more strongly bound than the $McrB_S$-$McrB_L$ heterodimer. To facilitate this strongly bound $McrB_L$ homodimer, we suggest that $McrB_L$ proteins contain multiple binding sites for dimerization and that some of these binding sites are lacking on the shorter $McrB_S$.

Antisense RNA and the proposed model. Data from antisense RNA experiments can now be evaluated with reference to the proposed model for McrBC active restriction complex formation and regulation. It is thought that ASR1 simply caused a decrease in active complex formation by lowing net $McrB_L$ homodimer produced. ASR3 produced lowered expression of *mcrC* and, likewise, lowered active complex formation by limiting available McrC from the active $McrB_L$ homodimer. If this were true, then $McrB_L$ homodimer levels should persist and this host should maintain McrB* restriction levels in the presence of ASR3. This proved to be true. JM107 (pASR3) showed decreased McrBC restriction while showing no loss of McrB* restriction. This suggests that ASR3 lowered McrC production only

and had no effect on *mcrB* expression. This is also the first report of measurable McrB* activity from a chromosomally encoded *mcrB* gene.

The most dramatic effects observed using antisense RNA were observed using ASR2. It was unknown what effect, if any, lowered $McrB_S$ amounts would have on restriction. Two effects readily evident were: (1) variable restriction levels, and (2) slow growing cells that lysed upon serial transfer in broth medium. We believe that both of these effects are explained by the lowered $McrB_S$ levels. It is thought that since less $McrB_S$ is available to produce inactive complexes, the number of active complexes increases (Fig. 23) causing dramatically increased restriction. This restriction activity is postulated to be so excessive that low-level cleavage of non-methylated DNA, which is tolerated by the cell under normal conditions, is magnified beyond the ability of the cell to repair the damage. Chromosomal DNA damage occurs and the SOS response is triggered. SOS response was triggered by ASR2 as measured experimentally. This condition leads to non-septated cells that readily lyse in broth cultures when shaken. Restriction is lost because this extremely deleterious condition is selected against during this highly mutagenic condition. Restriction defective strains are selected for and restriction is concomitantly measured as low. The variable levels of restriction observed under certain conditions is explained by a changing cell population as restriction proficient cells are selected against and displaced by restriction defective cells. One way to test this result would be to cure JM107 (pASR2) of this plasmid and measure restriction. It should be seen that the host remains restriction negative.

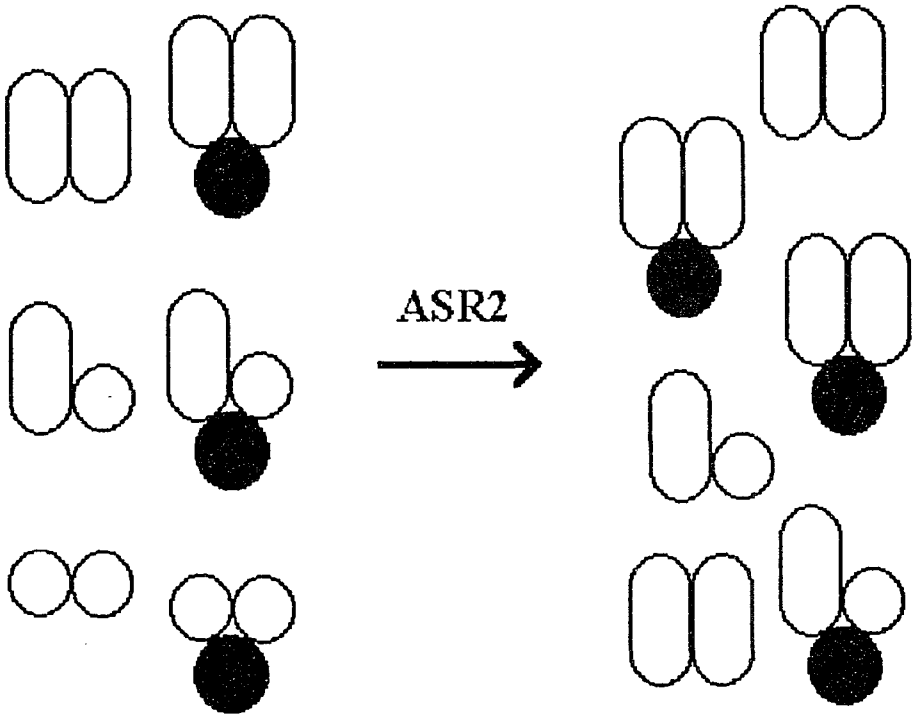


Fig. 23 - Decreasing $McrB_S$ synthesis using ASR2 may increase the proportion of active complexes. The ovoid represents $McrB_L$ and the open circle represents $McrB_S$. The filled circle represents McrC. This model shows the effect of lowering the number of $McrB_S$ subunits.

Hyper-restriction and the model for McrBC. Data obtained using $McrB_L$ truncations can be interpreted using the model. It is thought that the active restriction complex is a $McrB_L$ homodimer and that $McrB_S$ causes the formation of inactive heterodimers. When $McrB_S$ is sufficiently truncated, it performs two functions to produce hyper-restriction: (1) Excess truncated $McrB_S$ binds to authentic $McrB_S$ to eliminate its ability to bind $McrB_L$ and produce inactive complexes. (2) Truncated $McrB_S$ binds $McrB_L$ to block $McrB_S$ binding, but still allow, due to the greatly diminished size of the truncated proteins, $McrB_L$ dimerization to form the active complex. The truncated $McrB_S$ can block the binding site on $McrB_L$ necessary for $McrB_S$ binding but $McrB_L$ can still dimerize possibly through the additional binding sites postulated to exist in the N-terminal end of $McrB_L$ (Fig. 24). This has the net effect of allowing almost all $McrB_L$ monomers to be used in active complex formation. Most $McrB_S$ is removed from circulation because it is bound by the truncated $McrB_S$. Truncated forms of $McrB_S$ from pBAB74 and pBAB75 are insufficient to block $McrB_L$-$McrB_L$ homodimer formation and, thus, allow hyper-restriction.

Restriction rescue was taken to represent interaction between $McrB_S$ and McrC. The longer truncated proteins $McrB_S271$ and $McrB_S181$ contain an adequate amount of $McrB_S$ to continue to interact with McrC. Of 298 $McrB_S$ amino acids, 234 are truncated to produce $McrB_S65$. $McrB_S65$ has lost its ability to interact with McrC. The same is true of $McrB_S35$. It is interesting to note that both $McrB_S65$ and $McrB_S35$ elicit hyper-restriction in the wild-type background. The failure of these truncated proteins to interact with McrC is unlikely to be the cause of hyper-

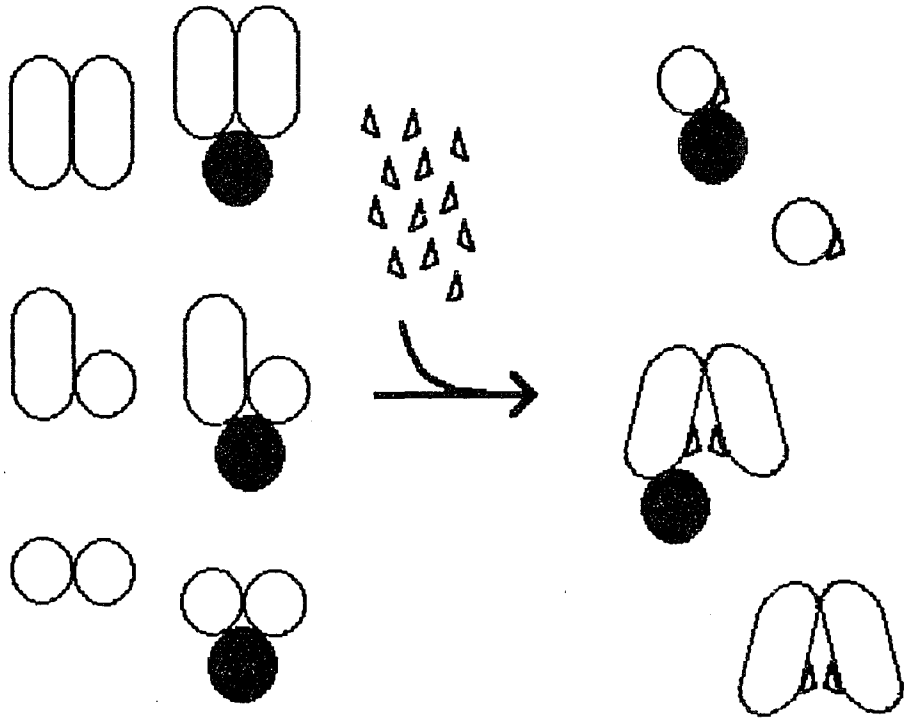


Fig. 24 - Truncated $McrB_S$ peptides allow hyper-restriction. The ovoid represents $McrB_L$ and the open circle represents $McrB_S$. The triangle represents truncated $McrB_S$, and the filled circle represents McrC. It is thought that truncated $McrB_S$ peptides cause hyper-restriction by permitting greater numbers of active complexes to be formed. When the smallest truncated $McrB_S$ is present, authentic $McrB_S$ will be removed from competition freeing essentially all $McrB_L$ to form active complexes.

restriction. This is true because hyper-restriction affects the McrB* assay results for DH5αMCR (pBAB43, pBAB75). In this strain, inability of $McrB_S35$ to interact with McrC is irrelevant since it possesses no McrC.

It is interesting to note that restriction of λ.O increased slightly when assaying DH5αMCR (pBAB43, pBAB75) for McrB* restriction. It should be noted that McrC is not present and that other hyper-restrictors where McrC was present showed no such gain in restriction of λ.O. This suggests that a hyper-restricting host in the absence of McrC can restrict DNA lacking traditional McrBC restriction recognition sites. It may be that non-methylated target sites are cleaved or that non-traditional methylated sites are seen as targets and cleavage occurs. This restriction of λ.O in the absence of McrC suggests that McrC is indeed a specificity subunit and, when lacking in a hyper-restricting strains, allows significant restriction at non-traditional sites. This cleavage may always occur in $mcrB^+C^-$ mutants but may not be measurable. In hyper-restricting strains, elevated levels of restriction permitted one to measure methylation-independent restriction. This suggests that McrC heightens specificity of the restriction complex for traditional McrBC recognition sites.

Binding studies with McrBC. According to the "caging effect" model (28), the polyacrylamide gel matrix stabilizes protein-DNA complexes by providing a cage so that dissociated protein components cannot diffuse away from the DNA throughout the 2-3 hour run time and instead undergo equilibrium binding. This model assumes that the protein remains active throughout this run time. This was not believed to be so with the McrBC complex. McrBC is known to be highly labile

and may become inactive shortly after entering the gel. Because so little gel shift was seen, it became necessary to cross link the protein-DNA complex using UV light. Protein-DNA complexes created during the exposure to UV light were joined covalently. Upon loading onto the gel, these protein-DNA complexes migrate more slowly than unbound DNA fragments and, therefore, can be separated as a measurable band. Approximately 15 minutes of UV exposure was seen to be adequate to produce this cross linked complex. This assay was performed to see which of the McrBC subunits bound DNA. It was found that none could specifically bind alone and that the full complex was required to bind the *Pvu*II methylated target fragments used.

Once results suggested that $McrB_S$ functioned as a regulator protein, models were put forth attempting to explain how it might accomplish this. One model, now disproven, suggested that $McrB_S$ alone or with McrC might bind DNA directly to block active complex access to some specific recognition and/or cleavage sites. It was found that $McrB_S$ alone or with McrC could not bind DNA. This was taken as evidence that $McrB_S$ regulated restriction by binding to or disrupting a multimeric restriction complex made up of at least $McrB_L$.

The optimum KCl concentration for specific binding was 50 mM. The results obtained from this assay suggested that Mg^{2+} is necessary for cleavage but not protein binding. We cannot rule out the possibility that binding may be tighter in the presence of Mg^{2+}. Another purportedly required cofactor is GTP although there is evidence to believe that ATP may function as a nucleotide cofactor of McrBC. Our results suggest that GTP allows protein-DNA binding and as the GTP

concentrations are lowered, the mobility of the McrBC-DNA band becomes altered. It is interesting to note that in the absence of added GTP, the addition of Mg^{2+} allows cleavage of the labeled target fragment. This may be because cellular GTP is already bound to an active McrBC complex.

Digestions of specifically methylated plasmids using crude extracts were used to test the necessity of ATP or GTP as a McrBC nucleotide cofactor. Our results suggest, at least at 0.3 mM conc., that ATP functions equally well as GTP to allow restriction. This result is in disagreement with recently published findings that suggest McrBC is a GTP-*dependent* nuclease (57, 76). Our results suggest that McrBC is a GTP-*utilizing* nuclease but that ATP may serve at some concentrations. In the Sutherland, *et al.* (76) study, ATP was never assayed alone and its purported inhibitive effect may only have been an effect of total purine nucleotides since GTP alone, at high concentrations, inhibited McrBC restriction (76).

During the McrBC-DNA UV cross linking experiment, it was seen that gel shift could not be obtained using extracts produced from logrithmically growing cells. It is known that the cell contains a concentration of 1 mM GTP and 3 mM ATP at this time (13). The ATP-GTP total of 4 mM is well within the range reported to result in inhibition of McrBC restriction (76). This could be an allosteric inhibition mechanism to keep McrBC restriction to a minimum during active DNA replication. We were able to produce the best gel shift results from extracts made from cells in stationary phase. This is the phase of growth when ATP and GTP concentrations would be low and thus allow McrBC restriction.

Crude extracts prepared from strains expressing various combinations of wild-type and mutated *mcrBC* genes were used to digest target DNA that possessed methylated *Pvu*II sites. We also found that the truncated products produced by pRAB11 (40 and 24-kDa) (65) could be complemented by McrC to cleave 5-hydroxymethylcytosine (^{hm5}C)-possessing DNA but not 5-methylcytosine (^{5m}C) possessing DNA. Extracts made from a pRAB12 (complete *mcrB*+ truncated McrC) possessing host were also adequate to restrict ^{hm5}C-containing DNA but not ^{5m}C-containing DNA. The McrBC system may be so finely tuned to restriction of ^{hm5}C-containing DNA that it will even cleave this DNA with significantly truncated $McrB_L$ (the restriction subunit) or McrC (the specificity subunit). Or ^{hm5}C-containing DNA of T4 may contain a diverse range of target nucleotide sequences, some of which serve as substrates for the modified forms of McrBC.

***In vitro* analysis of McrBC target sites.** In the McrBC-DNA UV cross linking assay, labeled DNA target fragment possessing three methylated *Pvu*II sites positioned 37-bp apart was primarily used. Specific McrBC-DNA bands could also be obtained using target fragments possessing two methylated *Pvu*II sites positioned 34-bp apart or with fragments with one methylated *Pvu*II site. These results are in disagreement with a recently proposed model (76) suggesting a minimum of two specific sites positioned 40 - 80-bp apart were necessary to define a specific McrBC restriction recognition site. Models for site recognition are limited by the sensitivity of the assay used to detect cleavage. It may be that the two site model holds true for the specific modified target and restriction cleavage assay used.

Evidence presented to support the two site model is substantial. However, it is important to realize that this model was developed using M.*Alu*I and M.*Msp*I methylated sites and may be correct with respect to these sites. Evidence suggests that even identical sites do not elicit DNA cleavage equally (76, this study). If this is so, elements in addition to the immediate methylation site must be necessary to define an optimal McrBC recognition and cleavage site. Our study was done using ^{m4}C-containing DNA produced by the *Pvu*II methylase. The ^{m4}C or the M.*Pvu*II pattern may require different elements to allow McrBC recognition and cleavage than does the M.*Alu*I methylase recognition site. The unique traits of the methylation pattern of our target DNA may elicit dissimilar results than that seen in the previous study. As our findings for defining a McrBC recognition and cleavage site differ from the proposed model, it is certain that additional work is needed to further define all elements involved. An interesting study may be done using the pBAB61 insert. This binding site is quite functional and the 170-bp fragment may contain additional sequences flanking the *Pvu*II site. Systematic deletions of nucleotides in the region flanking this site may yield insight into the true nature of McrBC recognition. Recently published results suggest that the McrBC cleavage site is proximal to the specific methylation site (76). Our results were in agreement as cleavage was achieved with the 131-bp target fragment of pBAB63.1.

In vitro DNA cleavage using crude extracts was used to analyze the methylation pattern required to produce a McrBC restriction recognition site. Two unique plasmids each possessing a single methylated M.*Pvu*II site were cleaved (Fig. 18). These results are in agreement with those obtained using target fragments

possessing a single, methylated *Pvu*II site for the McrBC-DNA UV cross linking assay. Together, these findings indicate that under the conditions used in this study, a single methylated site was sufficient for McrBC binding and target cleavage.

CONCLUSIONS

Significant findings presented in this work are the following:

(1) $McrB_S$ is a regulator or modulator of the McrBC restriction system. The levels of $McrB_S$ were found to affect the results of McrBC, McrB*, and Rgl restriction assays. Without functional $McrB_S$, hyper-restriction was observed and DNA damage resulting in the induction of the SOS response was documented.

(2) A working model was proposed in which the active restriction complex for McrBC and Rgl restriction minimally consists of a $McrB_L$ homodimer bound by McrC. $McrB_S$ forms inactive heterodimers with $McrB_L$ and disrupts active $McrB_L$ homodimers for McrB* restriction and $McrB_L$ homodimer-McrC complex for McrBC and Rgl restriction.

(3) Based on deletions of the C-terminal coding region of *mcrB*$_S$, domains of $McrB_S$ necessary for binding $McrB_L$ and McrC were identified.

(4) Data suggest that McrC is the sequence specificity subunit as hyper-restriction occurring without McrC present allowed low level restriction of DNA lacking a traditional restriction target for this system.

(5) Restriction occurred equally well at the concentrations tested using ATP or GTP as the nucleotide cofactor. GTP appears to be necessary for protein-DNA binding but Mg^{2+} does not.

(6) One M.*Pvu*II methylated site is adequate for McrBC binding and cleavage. Where two M.*Pvu*II methylation sites are present, binding can occur when they are as close as 34-bp.

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VITA

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Microbiology

Title of Dissertation: Characterization of the Subunits of the McrBC Restriction System in *E. coli* K12

Approved:

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